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There were two specific tasks listed in the proposal i.e. development of hPRL-G129R as a PRL receptor antagonist and development of PRL BP as PRL sequester as potential breast cancer therapeutics. The conclusion from this project are that we have demonstrated that hPRL-G129R has promise to be used as a breast cancer therapeutic, but no hPRL-BP. We have found that hPRL-G129R is able to inhibit breast cancer cell proliferation via the induction of apoptosis. We further presented evidence that shows that mechanism of hPRL-G129R induced breast cancer cell apoptosis is the regulation of Bcl-2/Bax gene expression. Data obtained from mouse breast cancer model also support the notion that is inhibition of PRL activity in breast cancer inhibit it growth. In addition, we explored the possibility of using hPRL-G129R as a targeting molecule by fusing it with proven effective anti-cancer agents such as immuno-modulators (IL-2) and angiogenesis inhibitors (endostatin). During past three years, we have published 10 manuscripts directly related to this subject. In summary, we have demonstrated that hPRL-G129R is a true PRL receptor antagonist. Its anti-breast tumor effects were confirmed using both in vitro and in vivo assays.

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Introduction

Human breast cancer is the most predominant malignancy with the highest mortality rate in women from western society. Many risk factors have been identified for this disease. Several lines of evidence strongly linked human prolactin (hPRL) to breast carcinogenesis. In this proposal, two novel approaches have been designed to generate hPRL receptor specific antagonists. First approach is to adopt a site-directed mutagenesis strategy by which hGH receptor antagonist, hGH-G120R, was discovered, to produce a mutated hPRL, hPRL-G129R, and use it as hPRL receptor **blocker**. The other approach is to design and produce a soluble form of extra-cellular domain of hPRL receptor namely hPRL binding protein (hPRL-BP), and use it to **sequester** autocrine/paracrine effects of hPRL. After cloning of hPRL and hPRL-BP cDNAs, mutation will be made in hPRL cDNA to generate hPRL-G129R. Human PRL, hPRL-G129R and hPRL-BP cDNAs will be produced and purified using *E. coli* protein expression system. The purified proteins will then be used to test its bioactivities in multiple human breast cancer cell lines and two non-breast origin human cancer cell lines (as controls) for receptor binding, inhibition of phosphorylation of the STATs protein induced by hPRL (as an indicator for intracellular signaling), and inhibition of human breast cancer cell proliferation. We hope that these two novel approaches will ultimately result in generation of hPRL antagonists that could be used to improve human breast cancer therapy.

Body

There were two specific tasks listed in the proposal i.e. development of hPRL-G129R as a PRL receptor antagonist and development of PRL BP as PRL sequester both are aimed as breast cancer therapeutics. Our conclusion from this project is that hPRL-G129R has promise to be used as a breast cancer therapeutic, but not hPRL-BP. We have found that hPRL-G129R is able to inhibit breast cancer cell proliferation via the induction of apoptosis. We presented evidence that shows the mechanism of hPRL-G129R induced apoptosis is through the regulation of Bcl-2/Bax gene expression. Based on the promising results obtained from hPRL-G129R, we further explored possibility of using hPRL-G129R as a targeting molecule by fusing it with proven effective cancer drugs as immuno-modulators (IL-2) and angiogenesis inhibitors (endostatin). During past three years, we have published 10 manuscripts directly related to this subject. In summary, we have demonstrated that hPRL-G129R is a true PRL receptor antagonist. Its anti-breast tumor effects were confirmed using both in vitro and in vivo assays. We are currently conducting pre-clinical studies of hPRL-G129R and hope to launch clinical phase studies.

Key Research Accomplishment

During past three years, we have published 10 manuscripts, 14 national meeting presentations (abstracts) and three patent filings directly related to this subject. In summary, we have demonstrated that hPRL-G129R is a true PRL receptor antagonist. Its anti-breast tumor effects were confirmed using both in vitro and in vivo assays. In addition, we have demonstrated that hPRL-G129R may be used as a targeting vehicle to produce breast cancer specific therapeutics. We are currently conducting pre-clinical studies of hPRL-G129R and hope to launch clinical phase studies in the near future.

Reportable Outcomes

Ten manuscripts:

1. Chen WY, Ramamoorthy P, Chen N, Sticca R, Wagner TE. A human prolactin antagonist, hPRL-G129R, inhibits breast cancer cell proliferation through induction of apoptosis. Clin. Cancer Res. 5, 3583-93, 1999.
2. Cataldo L, Chen NY, Li W, Wagner TE, Sticca RP and Chen WY. Inhibition of the Oncogene STAT3 by a Human Prolactin (PRL) Antagonist is a PRL Receptor Specific Event. Int. J. Oncology 17, 1179-1185, 2000.
3. Ramamoorthy P, Sticca RP, Wagner TE, Chen WY. In vitro Studies of a Prolactin Antagonist, hPRL-G129R, in human breast Cancer Cells. Int. J. Oncology 18, 25-32, 2001.
4. Beck MT, Holle H, Chen WY. Combination of PCR Subtraction and cDNA Microarray for Differential Gene Expression Profiling. Biotechniques 31, 1-4, 2001.
5. Peirce S and Chen WY. Quantification of Prolactin Receptor mRNA in Multiple Human Tissues and Cancer Cell Lines by Real Time RT-PCR J Endocrinol. 171, R1-4, 2001.
6. Chen NY, Li W, Cataldo L, Peirce S, and Chen WY. *In vivo* Anti-tumor Activities of a Human Prolactin Antagonist, hPRL-G129R. Int. J. Oncology 20, 813-818, 2002.
7. Zhang GR, Li W, Holle H, Chen NY and Chen WY. A novel design of targeted endocrine and cytokine therapy for human breast cancer. Clin. Cancer Res., 8:1196-1205, 2002.
8. Beck MT, Peirce SK and Chen WY. Regulation of *bcl-2* gene expression in human breast cancer cells by prolactin and its antagonist, hPRL-G129R. Oncogene 21, 5047-55, 2002.
9. Beck MT, Chen, NY, Franek K and Chen WY. Prolactin Antagonist Endostatin Fusion Protein as A Targeted Dual Functional Therapeutic Agent for Breast Cancer. Cancer Res., 63:3598-3604, 2003.
10. Peirce S and Chen WY. Human prolactin and its antagonist, hPRL-G129R, regulate *bax* and *bcl-2* gene expression in human breast cancer cells and transgenic mice. Oncogene (in press). 2003.

Fourteen Abstracts (National Meeting presentations).

1. A Human Prolactin Antagonist Modulates Transforming Growth Factors Beta-1 and Alpha in Human Breast Cancer Cells Ramamoorthy, P., Wagner, T.E. and Chen,W.Y. AACR 2000.
2. Inhibition of Oncogene STAT3 Phosphorylation by a Prolactin Antagonist, hPRL-G129R, in T-47D Human Breast Cancer Cells. L Cataldo, NY Chen, Q Yuan, W Li, P Ramamoorthy, TE Wagner, R Sticca and WY Chen. Endo 2000.
3. *In Vivo* Studies of the Anti-tumor Effects of a Human Prolactin Antagonist, hPRL-G129R in Nude Mice Chen, N.Y., Li, W., Cataldo, L., Sticca, R.P., Wagner, T.E. and Chen, W.Y. AACR 2001
4. A novel design of targeted endocrine and cytokine therapy for human breast cancer G.R. Zhang, W. Li[2], L. Holle, N.Y. Chen and W.Y. Chen. Endo 2001
5. Characterization of a human prolactin antagonist/ granulocyte macrophage colony stimulating factor fusion protein. L. Holle, W. Li, N.Y. Chen and W.Y. Chen. . Endo 2001
6. Profiling of apoptosis related genes responding to prolactin and its antagonist in human breast cancer cells. M.T. Beck, L. Holle and W.Y. Chen . Endo 2001
7. Real time RT-PCR analysis of relative prolactin receptor (PRLr) levels in human cancer cell lines. S.K. Peirce, R.B. Westberry and W.Y. Chen. . Endo 2001
8. Enhancement of the inhibitory effects of suppressor of cytokine signaling 3 (SOCS3) protein in cancer cells by VP22 Q. Yuan, S.K. Peirce, R.B. Westberry[2] and W.Y. Chen. . Endo 2001.
9. J. F. Langenheim, M.T. Beck, W.Y. Chen, From an antagonist back to an agonist: two wrongs do make a right. Endo 2002.
10. S. K. Peirce[1], M.T. Beck and W.Y. Chen. Regulation of bcl-2 expression by hprl and its antagonist, hprl-g129r, in human breast cancer cell lines. Endo 2002.
11. J.F. Langenheim and W.Y. Chen. Construction of human prolactin (hprl) receptor targeting fusion toxins for breast cancer treatment using prl antagonist and recombinant forms of pseudomonas exotoxin A. Endo 2002.
12. M.T. Beck and W.Y. Chen. Human prolactin antagonist and endostatin fusion protein for the treatment of breast cancer. Endo 2002.
13. Michele L. Scotti, Susan K. Peirce, Karl J. Franek, Wen Y. Chen. A Combination Approach Using Human Prolactin Antagonist and Herceptin for HER2-overexpressing Breast Cancer. AACR 2003.
14. Michele L. Scotti, John F. Langenheim and Wen Y. Chen. Generation of Human Prolactin Antagonist and Pro-Apoptotic Bik Fusion Protein as a Potential Targeted Breast Cancer Therapeutic. AACR 2003.

Three Patent Application Filings:

1. Bi-functional cancer treatment agents. Wen Y Chen and Thomas E. Wagner, Filed, March 22, 2000.
2. Multimeric ligands with enhanced stability. Wen Y Chen, Filed, April, 2002.
3. Human prolactin antagonist-angiogenesis inhibitor fusion proteins. Wen Y Chen, Filed, May, 2002.

Three Ph. D. students graduated, (Prevene Ramamoorthy, Mike Beck and Susan Peirce) partially supported through this award.

Conclusions:

Through the DoD funding, we have confirmed that that hPRL-G129R acted as a true hPRL receptor antagonist in human breast cancer cells both in vitro and in vivo. We have also made considerable progress in terms of designing hPRL-G129R based targeting therapeutics for breast cancer. This DoD idea award has brought opportunities to PI to fulfill his dreams of designing and testing novel anti-cancer drugs and actually see the ideas to become reality. The award also helped several graduate students in the lab to be able to complete their training in breast cancer research. The data generated through this award has resulted in ten manuscripts and many abstracts; part of the additional data has been used to generate new proposals for future funding. The PI would like to extent his sincere thanks to DoD for this award.

Appendices

Ten manuscripts

A Human Prolactin Antagonist, hPRL-G129R, Inhibits Breast Cancer Cell Proliferation through Induction of Apoptosis[1]

Wen Y. Chen,[2] Preveen Ramamoorthy, Nian-yi Chen, Robert Sticca, and Thomas E. Wagner

Oncology Research Institute, Cancer Center, Greenville Hospital System [W. Y. C., N-y. C., R. S., T. E. W.], and Department of Microbiology and Molecular Medicine, Clemson University [W. Y. C., P. R., T. E. W.], Greenville, South Carolina 29605

ABSTRACT

Human breast cancer is the predominant malignancy and the leading cause of cancer death in women from Western societies. The cause of breast cancer is still unknown. Recently, the association between human prolactin (hPRL) activity and breast cancer has been reemphasized. Biologically active hPRL has been found to be produced locally by breast cancer cells that contain high levels of PRL receptor. A high incidence of mammary tumor growth has also been found in transgenic mice overexpressing lactogenic hormones. More importantly, it has been demonstrated that the receptors for sex steroids and PRL are coexpressed and cross-regulated. In this study, we report that we have designed and produced a hPRL antagonist, hPRL-G129R. By using cell proliferation assays, we have demonstrated that: (*a*) hPRL and E2 exhibited an additive stimulatory effect on human breast cancer cell (T-47D) proliferation; (*b*) hPRL-G129R possessed an inhibitory effect on T-47D cell proliferation; and (*c*) when antiestrogen (4-OH-tamoxifen) and anti-PRL (hPRL-G129R) agents were added together, an additive inhibitory effect was observed. We further investigated the mechanism of the inhibitory effects of hPRL-G129R in four hPRLR positive breast cancer cell lines. We report that hPRL-G129R is able to induce apoptosis in all four cell lines in a dose-dependent manner as determined by the Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay. The apoptosis is induced within 2 h of treatment at a dose as low as 50 ng/ml. We hope that the hPRL antagonist could be used to improve the outcome of human breast cancer therapy in the near future.

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[2] To whom requests for reprints should be addressed, at Oncology Research Institute, Greenville Hospital System, 900 West Faris Road, Greenville, SC 29605. Phone: (864) 455-1457; Fax: (864) 455-1567; E-mail: wchen@ghs.org.

INTRODUCTION

Human breast cancer is the predominant malignancy and leading cause of cancer death in women from Western societies (1, 2). According to a recent estimation by the American Cancer Society, one in every eight women from the United States will develop breast cancer, and the disease will kill 43,500 women in 1998. The cause of breast cancer is still unknown, but its great rarity among males indicates an etiological role for the female sex hormones, whereas varying geographic distribution also points to the importance of environmental factors (2). Although generally slow growing, breast cancer develops invasive properties early in its pathogenic progression. By the time it has become clinically apparent, it is likely to have already metastasized to distant sites. It is this pattern that accounts for the failure of purely local treatment to control the disease. For decades, the primary therapy for women with breast cancer has been surgery or radiation or a combination of both (1, 2).

hPRL[3] is a neuroendocrine polypeptide hormone discovered nearly 60 years ago. It is primarily produced by the lactotrophs in the anterior pituitary gland of all vertebrates. The biological activities of PRL are mediated by specific membrane receptors, *i.e.,* PRLRs (3). On the basis of several conserved features (a single transmembrane domain and conserved amino acid sequences in the extracellular domain), PRLRs together with GH receptor, have been categorized into the cytokine receptor superfamily (3). The best-characterized action of PRL is on the mammary gland. In this organ, PRL plays a decisive role in the stimulation of DNA synthesis, epithelial cell proliferation, and the promotion of milk production (4). The generation of PRL (4) and PRLR (5) gene knock-out mice have unambiguously demonstrated that PRL and PRLR are the key regulators in mammary development.

Several lines of evidence strongly link hPRL to breast cancer development: (*a*) it has been reported that female hGH transgenic mice have a high incidence of breast cancer in contrast to sporadic cases found in bovine GH transgenics (6). The high incidence of breast cancer in hGH transgenic mice is believed to be attributable to the lactogenic activity of hGH, which is a unique feature of primate GHs. A recent report of breast cancer development in hPRL transgenic mice further confirmed the role of hPRL in the stimulation of breast cancer (7); and (*b*) the finding of hPRL mRNA in mammary tissues (8–10) and the detection of biologically active hPRL in human breast cancer cells (11) suggest that hPRL is produced locally as

[3] The abbreviations used are: hPRL, human prolactin; PRLR, PRL receptor; GH, growth hormone; hGH, human GH; bGH, bovine GH; RT-PCR, reverse transcription-PCR; ER, estrogen receptor; FBS, fetal bovine serum; IRMA, immunoradiometric assay; E2, estradiol; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

an autocrine/paracrine growth factor within the mammary glands. This extrapituitary production of hPRL might not cause detectable systemic change of hPRL in serum yet could exert significant local stimulatory effects (12). In support of this concept, it has also been reported that the expression levels of PRLRs are significantly higher in human breast cancer cells or in surgically removed breast cancer tissues than in normal breast epithelial tissues (13–15). The high levels of PRLRs in malignant breast tissue make these cells highly sensitive to stimulation by hPRL (15).

In our previous studies, we demonstrated that the third α-helix of GH is important for its growth-promoting activities (16–21). We further demonstrated that Gly 119 of bGH (18) or Gly 120 of hGH (19) plays a critical role in the action of GH in stimulating growth enhancement. The mechanism of these GH antagonists was further studied by other groups (22, 23). It is generally accepted that GH transduces its signal via a sequential receptor binding mechanism to form a one hormone-two receptor complex (22, 23). Receptor dimerization is thought to be a key step for GH signal transduction. Any amino acid substitution (other than Ala), especially one with a bulky side chain such as Arg at position 120 of hGH, will prevent receptor dimerization, resulting in a GH antagonist (16–21). As a member of the GH family, hPRL is believed to share a signal transduction mechanism similar to GH (24–27). It is, therefore, reasonable to predict that if a key amino acid within the third α-helix of hPRL is substituted, it may be possible to produce a hPRL-specific antagonist in much the same manner that hGH antagonists have been produced.

In this paper, we report that by adopting a strategy similar to that which we used in designing the GH antagonist, we have developed a hPRL antagonist in which a Gly residue at position 129 was substituted with Arg (hPRL-G129R). We have demonstrated the following three hPRL-related findings: (*a*) single amino acid substitution mutation at position 129 of hPRL (hPRL-G129R) resulted in a hPRL antagonist, confirmed by cell proliferation assays; (*b*) when hPRL-G129R was applied together with 4-OH-tamoxifen, an additive inhibitory effect was observed; and (*c*) the inhibitory effect of hPRL-G129R on human breast cancer cells is through the induction of apoptosis. We believe that development of the hPRL-G129R, a hPRL antagonist, might open a new avenue in the design of adjuvant therapy to improve the treatment of breast cancer.

Table 1 Comparison of amino acid sequences within the third α-helical region among PRLs (42)[a]

		129
Human	PRL	IEEQTKRLLR **G** MELIVS-QVHP
Rat	PRL	IEEQNKRLLE **G** IEKIIG-QAYP
Mouse	PRL	IEEQNKQLLE **G** VEKIIS-QAYP
Hamster	PRL	IGEQNKRLLE **G** IEKILG-QAYP
Fin whale	PRL	EEEENKRLLE **G** MEKIVG-QVHP
Mink	PRL	IEEENRRLLE **G** MEKIVG-QVHP
Cattle	PRL	IEEQNKRLIE **G** MEMIFG-QVIP
Sheep	PRL	EEEENKRLLE **G** MENIFG-QVIP
Pig	PRL	IEEQNKRLLE **G** MEKIVG-QVHP
Camel	PRL	IEEQNKRLLE **G** MEKIVG-QVHP
Horse	PRL	EIEQNRRLLE **G** MEKIVG-QVQP
Elephant	PRL	VKEENQRLLE **G** IEKIVD-QVHP
Ancestral mammal	PRL	IEEENKRLLE **G** MEKIVG-QVHP
Chicken	PRL	IEEQNKRLLE **G** MEKIVG-RVHS
Turkey	PRL	IEEQDKRLLE **G** MEKIVG-RIHS
Sea turtle	PRL	IEEQNKRLLE **G** MEKIVG-QVHP
Crocodile	PRL	IEEQNKRLLE **G** MEKIIG-RVQP
Alligator	PRL	IEEQNKRLLE **G** MEKVIG-RVQP
Ancestral amniote	PRL	IEEQNKRLLE **G** MEKIVG-QVHP
Xenopus	PRL	VEEQNKRLLE **G** MEKIVG-RIHP
Bullfrog	PRL	VEEQTKRLLE **G** MERIIG-RIQP
Lungfish	PRL	VEDQTKQLIE **G** MEKILS-RMHP
Tilapia	PRL	MQQYSKSLKD **G** LD-VLSSKMGS
Tilapia	PRL	MQEHSKDLKD **G** LD-ILSSKMGP
Common carp	PRL	LQENINSLGA **G** LEHVF-NKMDS
Bighead carp	PRL	LQDNINSLGA **G** LERVV-HKMGS
Silver carp	PRL	LQDNINSLVP **G** LEHVV-HKMGS
Chum salmon	PRL	LQDYSKSLGD **G** LD-IMVNKMGP
Chinook salmon	PRL	LQDYSKSLGD **G** LD-IMVNKMGP
Trout	PRL	LQDYSKSLGD **G** LD-IMVNKMGP
		120
Human	GH	VYDLLKDLEE **G** IQTLMRELEDG
Bovine	GH	VYEKLKDLEE **G** ILALMRELEDG

[a] Two GH sequences are also included. Gly 129 of hPRL is in bold.

MATERIALS AND METHODS

RT-PCR

The RT-PCR technique was used to clone hPRL cDNA. Human pituitary mRNA was purchased from Clontech Laboratory, Inc. (Palo Alto, CA). A RT-PCR kit was from Perkin-Elmer, Inc. (Norwalk, CT). The hPRL antisense primer (for the reverse transcriptase reaction) was designed 2 bases from the stop codon (shown in boldface) of hPRL cDNA (5′-GC**TTA**G-CAGTTGTTGTTGTG-3′), and the sense primer was designed from the translational start codon ATG (5′-**ATG**AACAT-CAAAGGAT-3′). The RT-PCR reaction was carried out following the manufacturer's recommendation. The PCR product was then cloned into an expression vector pCDNA3.1 from Invitrogen Corp. (Carlsbad, CA). The expression of hPRL cDNA was controlled by the human immediate-early cytomegalovirus enhancer/promoter and a polyadenylation signal and transcription termination sequence from the *bGH* gene. This vector also contains a neomycin gene that allows for selection of neomycin-resistant mammalian cells.

Rational Design of hPRL-G129R

We have compared the amino acid sequences of all known PRLs in the third α-helical region and aligned them with GH sequences (Table 1). It is clear that Gly 129 of hPRL is invariable among PRLs and corresponds to hGH 120, suggesting a potentially important role in its function. We, therefore, decided to make a single amino acid substitution mutation at Gly 129 of hPRL (hPRL-G129R). We have used a similar approach to that which we have used successfully previously in the discovery of hGH antagonists in the hope of producing a hPRLR-specific antagonist (Fig. 1).

Oligonucleotide-directed Mutagenesis

hPRL-G129R cDNA was generated using a PCR mutagenesis protocol. Oligonucleotides containing the desired mutation (5′-CTTCTAGAGCGCATGGAGCTCATA-3′ and 5′-CCCT-CTAGACTCGAGCGGCCGCC-3′) were synthesized by National Biosciences, Inc. (Plymouth, MN). The codon for 129 Arg is in boldface, and the restriction site *Xba*I is underlined.

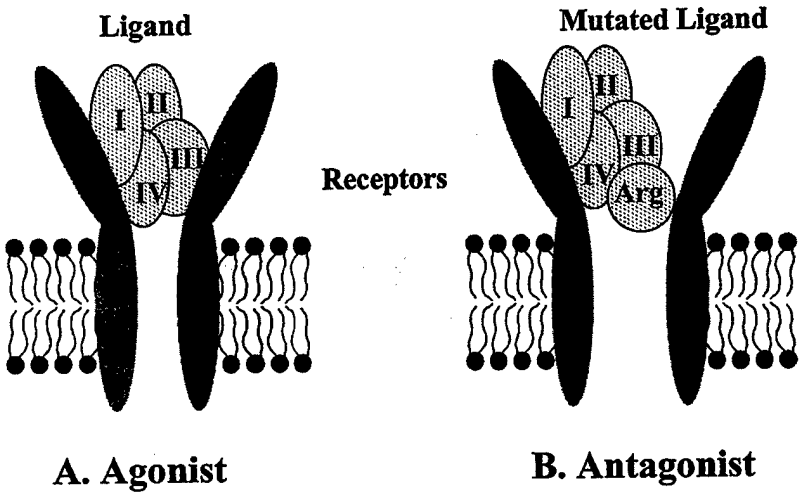

Fig. 1 Schematic illustration of the mechanism of GH or hPRL (ligand) antagonist. Four helical regions in the ligand (*dotted ovals*) are labeled as *I, II, III,* and *IV*. Two membrane bound receptors (*shaded dark ovals*) are also shown in the figure. *Arg,* substitution mutation in the third α-helix, resulting in hindering a second receptor to form a functional complex (from *A* to *B*).

The PCR product was digested with *Xba*I and ligated back into the vector described previously. The mutation was then confirmed by DNA nucleotide sequencing.

Human Breast Cancer Cell Lines

The human breast cancer cell lines used in this study are MDA-MB-134, T-47D, BT-474, and MCF-7 from the American Type Culture Collection. These human breast cancer cell lines have been characterized as ER-positive and PRLR-positive cell lines (28). T-47D and BT-474 cells were grown in RPMI 1640 (phenol red free to avoid its potential estrogen-like activities) supplemented with 10% FBS (Life Technologies, Inc.) and American Type Culture Collection recommended supplements. MCF-7 cells were grown in DMEM (phenol red free), supplemented with 10% FBS. The cells were grown at 37°C in a humid atmosphere in the presence of 5% CO_2. The MDA-MB-134 cells were grown in Leibovitz's L-15 medium supplemented with 20% FBS and grown in a CO_2-free atmosphere.

Expression and Production of hPRL and hPRL-G129R Proteins

Mouse L-cell transfection and stable cell selection were performed as described previously with minor modification (29). Briefly, cells were plated in a six-well plate and cultured until the culture was 50% confluent. On the day of transfection, cells were washed once with serum-free medium and cultured in 1 ml of serum-free medium containing 1 μg of pcDNA3-hPRL or pcDNA3-hPRL-G129R and 10 μl of LipofectAmine (Life Technologies, Inc.) for 5 h. Two ml of growth medium were added to the DNA/LipofectAmine solution, and incubation continued. After 18–24 h of incubation, fresh growth medium was used to replace the medium containing DNA/LipofectAmine mixture. At 72 h after transfection, cells were diluted 1:10 and passed into the selective medium (400 μg/ml G418) to select for *neo* gene expression. Individual colonies were isolated and expanded. The expression levels of the individual cell lines were determined by using an IRMA kit from Diagnostic Products Corp. (Los Angeles, CA). The cell lines with high expression levels were expanded.

Conditioned medium containing hPRL and hPRL-G129R was prepared as follows. Stable cells were plated in T-150 culture flasks at 85–90% confluence. The growth medium were then replaced with 50 ml of RPMI 1640 containing 0.5% dextran-coated charcoal-FBS and collected every other day for three times. The collected media were then pooled and filtered through a 0.22 μm filter units to remove cell debris and stored at −20°C until use. The concentration of hPRL or hPRL-G129R was determined by hPRL IRMA. Each batch product was further verified using a Western blot analysis protocol (30). We have used this protocol in hGH analogue studies, including hGH antagonists, for *in vitro* studies (19).

Radioreceptor Binding Assay

hPRLR binding assays were performed as described previously (19, 31). Briefly, T-47D cells were grown in six-well tissue culture plates until 90% confluent (~10^5 cells/well). Monolayers of cells were starved in serum-free RPMI 1640 medium for 2 h. The cells were then incubated at room temperature in serum-free RPMI 1640 containing 8×10^4 cpm ^{125}I-labeled hPRL (specific activity, 30 μCi/μg; NEN DuPont, Boston, MA) with or without various concentrations of hPRL (from NIH as standard) and hPRL-G129R. Cells were then washed three times in serum-free RPMI 1640 and solubilized in 0.5 ml of 0.1 N NaOH/1% SDS, and the bound radioactivity was determined by a gamma counter (model 4/600plus; ICN Biomedical, Costa Mesa, CA). EC_{50}s of hPRL and hPRL-G129R were then determined and expressed as mean ± SD. Comparison was made by Student's *t* test.

Human Breast Cancer Cell Proliferation Assays

hPRL-G129R Conditioned Media. The assay conditions were modified from that described by Ginsburg and Vonderharr (11). T-47D cells were trypsinized and passed into 96-well plates in RPMI 1640 containing 0.5% FBS that was treated with charcoal/dextran-treated FBS (Hyclone, Logan, UT) in a volume of 100 μl/well. The optimal cell number/well for each cell line was predetermined after titration assay. We have found that 15,000 cell/well are optimal for T-47D cells.

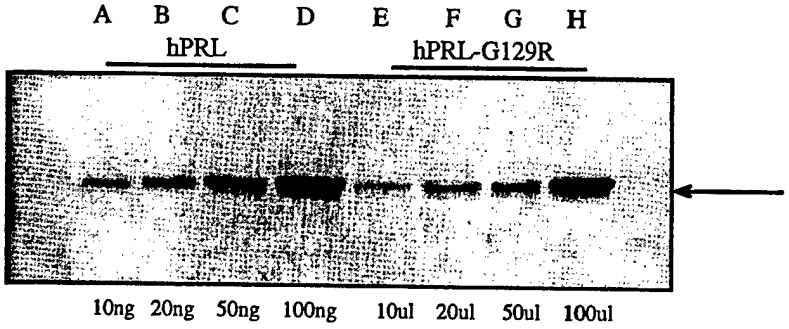

Fig. 2 Immunoblot analysis of the hPRL-G129R gene expression by mouse L cells. A polyclonal rabbit anti-hPRL (1:500; Biodesign International, Kennebunk, ME) was used as primary antibody, and a goat anti-rabbit IgG horseradish peroxidase conjugate (1:500; Boehringer Mannheim) was used as secondary antibody. *Lanes A–D*, samples containing purified hPRL (from NIH) as standards. *Lanes E–H*, culture media from stably transfected mouse L cells.

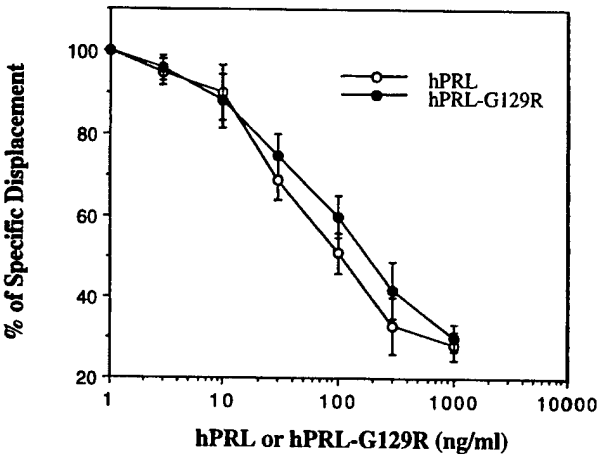

Fig. 3 Competitive radioreceptor binding assays. The data from triplicate determinations of three separate experiments are presented as the means; *bars*, SD. *Ordinate*, hPRL or hPRL-G129R concentrations. *Abscissa*, percentage of displacement of the total binding. EC_{50}s were determined and compared using Student's *t* test. There was no significant difference between the two EC_{50}s ($P > 0.05$).

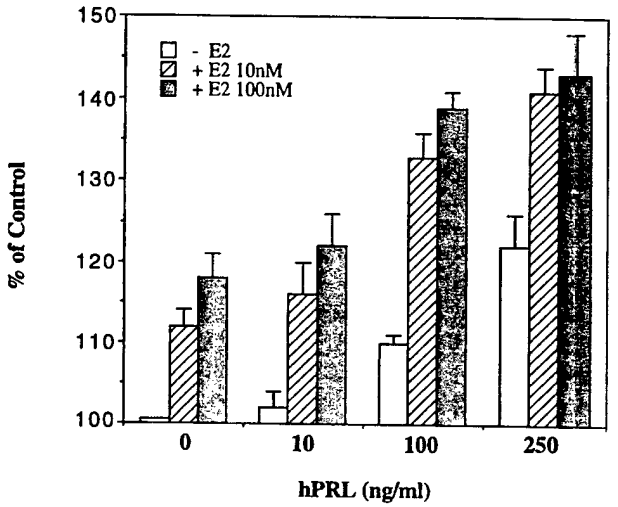

Fig. 4 Dose-response effects of hPRL and its additive effects with E2 in T-47D human breast cancer cell proliferation assay. *X axis*, the hPRL concentration either in the absence (□) or presence of E2. Each data point represents a mean of at least three independent experiments with triplicate wells; *bars*, SD.

The cells were allowed to settle and adhere overnight (12–18 h), and subsequently various concentrations of either hPRL, hRPL-G129R, E2, or 4-OH-tamoxifen in a total volume of 100 μl of culture media were added. Purified hPRL (kindly provided by Dr. Parlow, National Hormone and Pituitary Program, NIH, Bethesda, MD) was used as a positive control for hPRL produced from stable L cells. Cells were incubated for an additional 96 h at 37°C in a humidified 5% CO_2 incubator. After incubation, MTS-PMS solution (Cell Titer 96 Aqueous kit; Promega Corp.) was added to each well, following the manufacturer's instructions. Plates were read at 490 nm using a Bio-Rad benchmark microplate reader. The experiments were carried out in triplicates and repeated three to six times for each cell line.

Coculture Experiments. This design of the cell proliferation assay is to take advantage of stable mouse L cell lines we have established that produce hPRL and hPRL-G129R. Increasing numbers of L cells (or L-hPRL or L-hPRL-G129R cells) in a range of 4,500–27,000 cells/well were cocultured with fixed number of T-47D (9,000/well) in 96-well plates. At the same time, a correspondent set of L cells (or L-hPRL or L-hPRL-G129R cells) was cultured in the same plate (without coculture with T-47D) as background controls. The total volume of the coculture was 200 μl. The concentrations of hPRL or hPRL-G129R at the end of 72-h coculture were measured at 20–200 ng/ml, which is within the physiological range and is similar to that of the conditioned media experiments. After incubation, MTS-PMS solution was added to each well at 24, 48, or 72 h (best response was observed at 72 h and reported in this paper). Plates were then read at 490 nm using a Bio-Rad benchmark microplate reader. The absorbance (*A*) of T-47D cells was calculated as total *A* (*A* of T-47D plus L, L-hPRL or L-hPRL-G129R cells, respectively) minus the background *A*s (L, L-hPRL, or L-hPRL-G129R cells alone).

TUNEL Assay

This assay (Fluorescein Apoptosis detection system; Promega Corp.) works by labeling the nicks of the fragmented DNA at the 3-OH ends. The fluorescein-labeled dUTP is incorporated at the 3-OH ends by terminal deoxynucleotidyl transferase. Four human breast cancer cell lines were used in this study. Before the assay, the breast cancer cells were switched to 10% charcoal/dextran-treated FBS (CCS) for a week. Subsequently, the cells were plated onto an eight-chambered slide

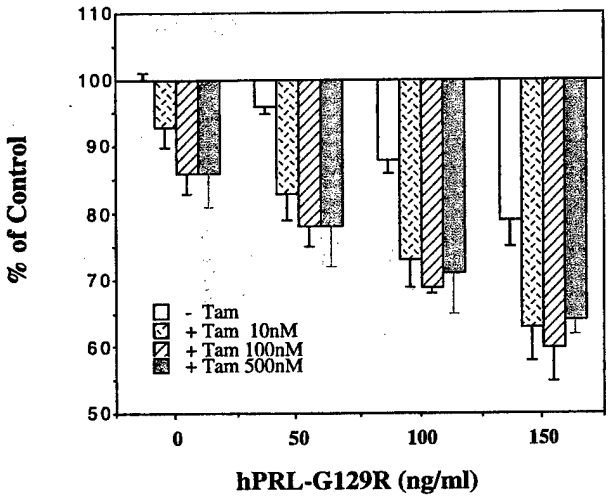

Fig. 5 Dose-response inhibitory effects of hPRL-G129R and its additive effects with 4-OH-Tamoxifen in T-47D human breast cancer cell proliferation assay. *X axis,* the hPRL-G129R concentration either in the absence (□) or presence of 4-OH-Tamoxifen. Each data point represents a mean of at least three independent experiments with triplicate wells; *bars,* SD.

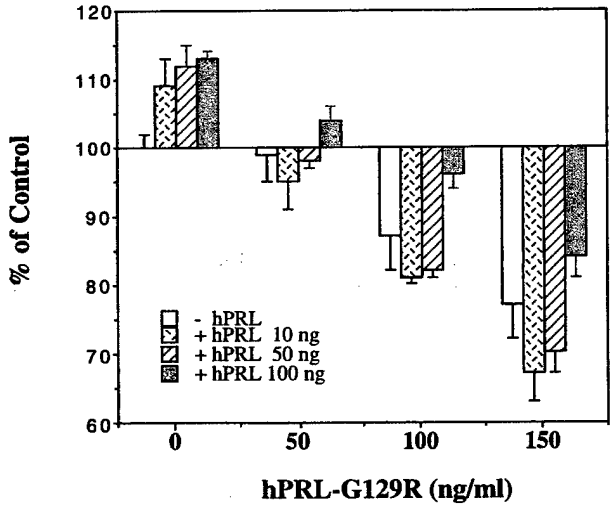

Fig. 6 Dose-response inhibitory effects of hPRL-G129R on hPRL-induced T-47D cell proliferation. *X axis,* concentration of hPRL-G129R either in the absence of hPRL (□) and the presence of hPRL. Each data point represents a mean of at least three independent experiments with triplicate wells; *bars,* SD.

system (Lab TekII) at a confluence of 60–70% per chamber. The next day, the breast cancer cells were treated with various concentrations of hPRL-G129R in conditioned medium (0.5% CCS) or 4-OH-Tamoxifen (in 0.5% CCS containing growth medium). To demonstrate the specificity of the antagonist, hPRL-G129R was also either mixed with PRL or with polyclonal anti-hPRL antibodies (kindly provided by Dr. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases) before being applied to breast cancer cells. In the case of

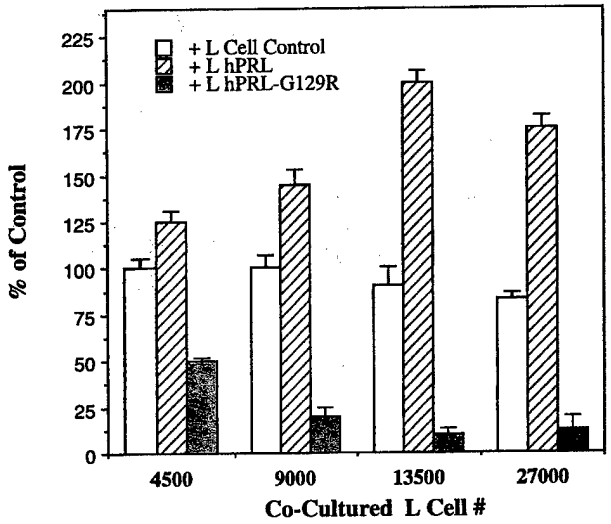

Fig. 7 Dose-response inhibitory effects of hPRl-G129R in T-47D human breast cancer cells using the coculture method. *X axis,* the cocultured L cell (control, L-PRL, or L-hPRL-G129R) numbers. Each data point represents a mean of at least three independent experiments with triplicate wells; *bars,* SD.

anti-hPRL antibody experiments, 125 ng/ml of hPRL-G129R were preincubated with anti-hPRL antibodies for 6 h at 4°C before adding to the cells. After the assigned period of treatment, the chambers were dismantled, and the assay was performed as per the manufacturer's instructions. The slides were examined under a FITC filter using an Olympus IX 70 microscope system.

RESULTS

Cloning and Mutagenesis of hPRL

hPRL cDNA was cloned from human pituitary mRNA using the RT-PCR technique. The size of the corresponding PCR product was 663 bp in length (data not shown), and it was cloned into the pcDNA 3.1 expression vector. The nucleotide sequence of hPRL was determined by the dideoxy chain-termination method using an automatic sequencer (PE Applied Biosystems, Foster City, CA). The hPRL cDNA sequence was found identical to that reported in GenBank, except for one base difference that results in a silent mutation at codon 21 (CTG→]CTC). hPRL-G129R cDNA was also generated by PCR and sequenced.

Expression of hPRL and hPRL-G129R

Mouse L cell were stably transfected with either hPRL or hPRL-G129R cDNAs, and neo-resistant clones were selected and expanded. Conditioned media were collected and tested for expression by use of an IRMA kit. We have generated hPRL and hPRL-G129R stable mouse L-cell lines that produced hPRL and hPRL-G129R in a quantity of ~1 mg/l every 24 h/million cells (Fig. 2).

Radioreceptor Binding Assay

The assay was carried out in a homologous system using ^{125}I-labeled hPRL in the presence or absence of various con-

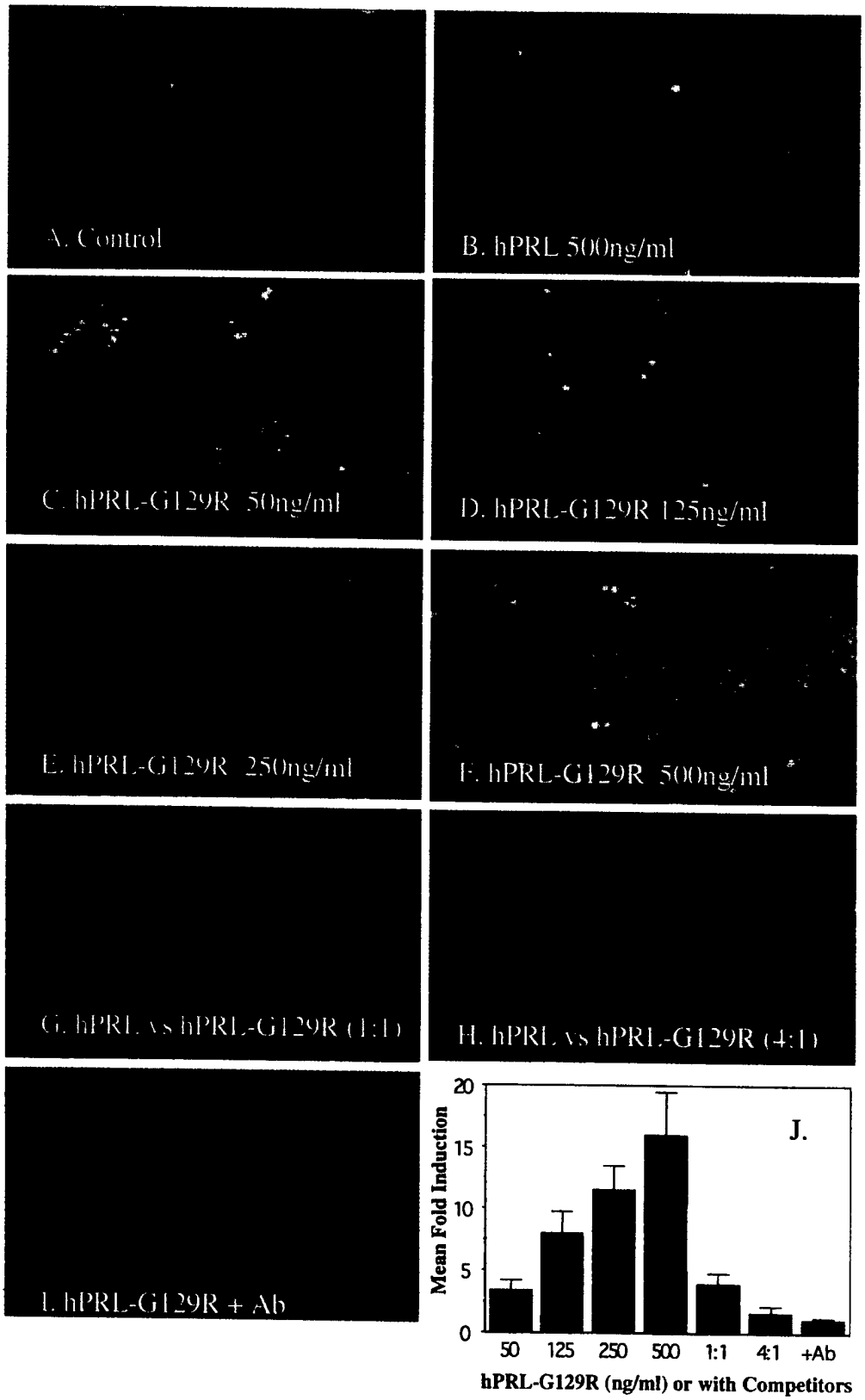

Fig. 8 Dose-response of T-47D human breast cancer cells to hPRL-G129R after 24 h of treatment using the TUNEL assay (*A–F*). *G* and *H*, results of competition between hPRL and hPRL-G129R at 1:1 ratio (125 ng/ml of each; *G*) and 4:1 ratio (500 ng/ml hPRL + 125 ng/ml hPRL-G129R; *H*). *I*, result of anti-hPRL antibody pretreatment (125 ng/ml of hPRL-G129R in 100-μl volume + 100-μl antiserum). *J*, quantification of the same experiment (fold induction of apoptotic cells/field over control; average of three measurements).

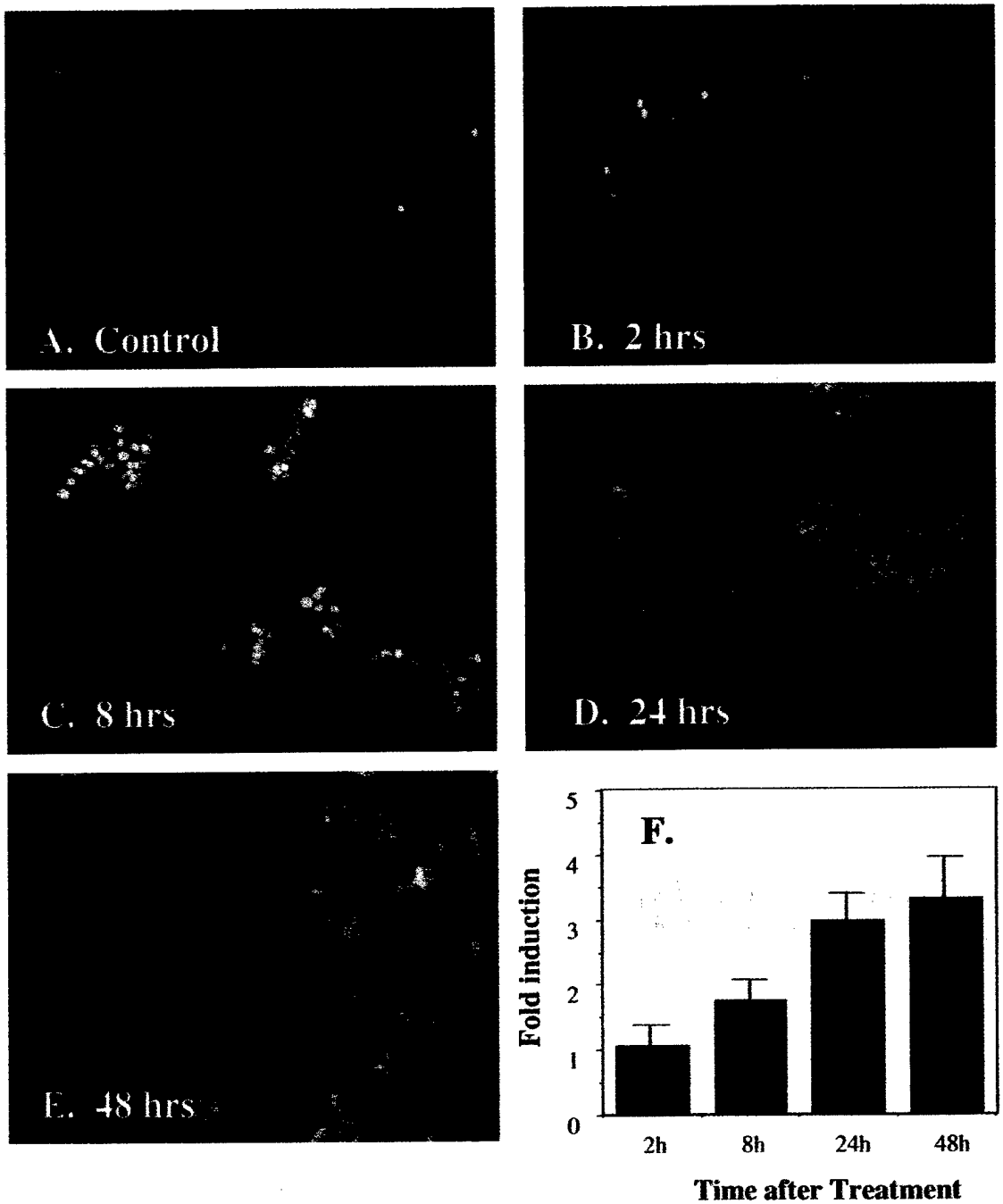

Fig. 9 Time course of T-47D human breast cancer cells responding to hPRL-G129R treatment (50 ng/ml) using the TUNEL assay (*A–E*). *F*, quantification of the same experiment (fold induction of apoptotic cells/field over control; average of three measurements).

centrations of unlabeled hPRL or hPRL-G129R and T-47D cells. The results demonstrated that there was no significant change in EC_{50}s ($P > 0.05$) of hPRL-G129R (3.01 nM ± 0.24 nM) as compared with hPRL (1.89 ± 0.18 nM; Fig. 3). These results were similar to our previous studies regarding bGH antagonist (bGH-G119R; Ref. 16) and hGH antagonist (hGH-G120R; Ref. 19).

Human Breast Cancer Cell Proliferation Assays

Conditioned Media. Human PRL and hPRL-G129R were tested further for its ability to stimulate/inhibit breast cancer cell proliferation in cell culture. Ninety-six-well cell proliferation assay results are shown in Figs. 4–6. hPRL stimulated T-47D proliferation in a dose-dependent manner. The maximum stimulation of hPRL (250 ng/ml) was ~20% over

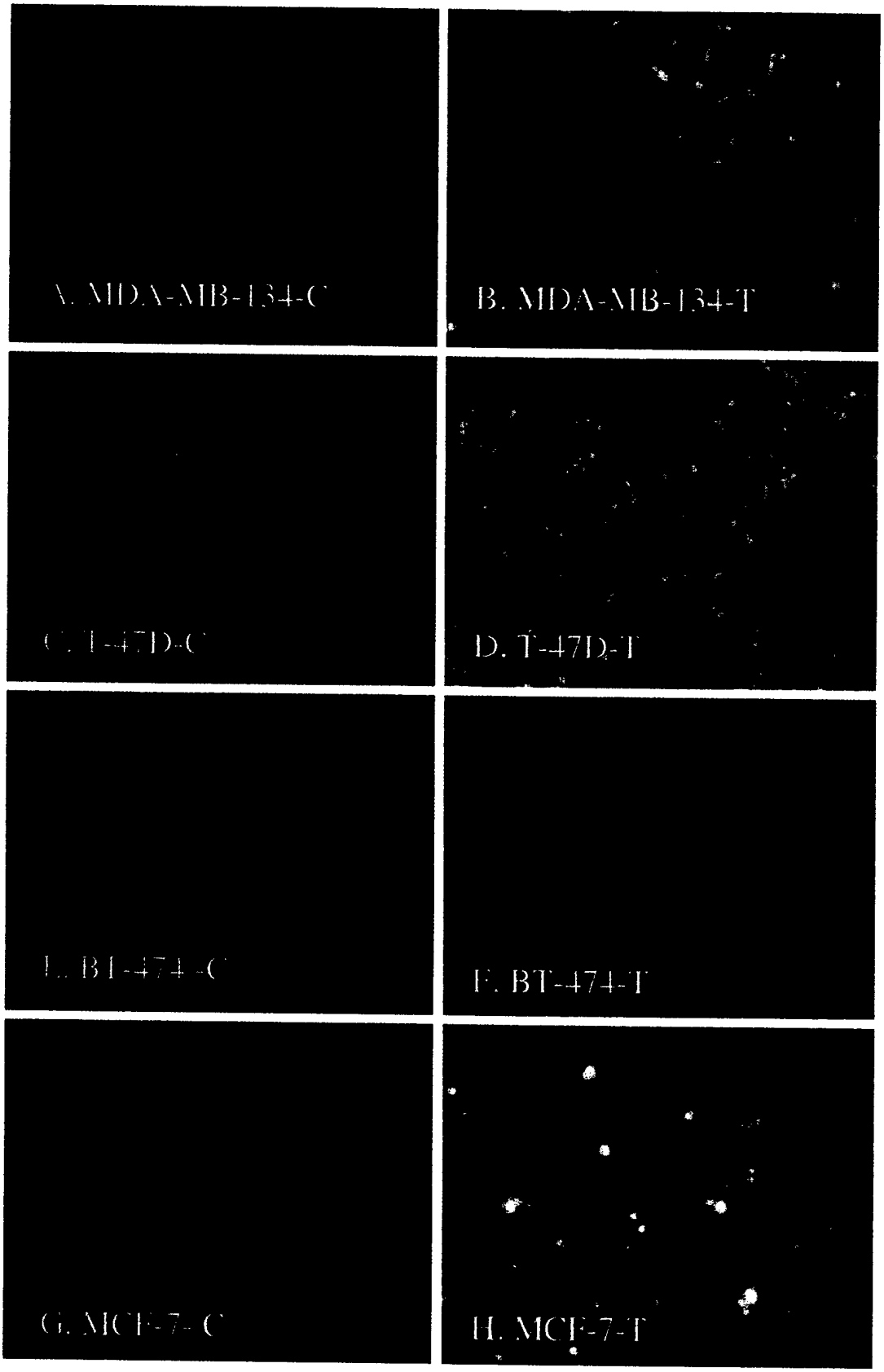

Fig. 10 Response of multiple breast cancer cells (as labeled) to treatment with 250 ng/ml hPRL-G129R for 24 h using the TUNEL assay. *-C*, control cells; *-T*, treated cells.

basal levels after a single dose/4-day incubation. However, when hPRL and E2 were applied simultaneously, an additive effect was observed. The maximum response of hPRL (100 ng/ml) in the presence of 10 nM of E2 was more than tripled as compared with hPRL alone (Fig. 4).

hPRL-G129R, on the other hand, exhibited dose-dependent inhibitory effects on cell proliferation (Fig. 5, □). It is noteworthy to point out that the inhibitory effect of hPRL-G129R (150 ng/ml) was more potent than the maximal 500 nM dose of 4-OH-Tamoxifen in our assay system (Fig. 5). The maximum inhibition of a single dose of 4-OH-Tamoxifen (500 nM) is ~85% of control, whereas the maximum inhibition by a single dose of hPRL-G129R resulted in 75% of control. More importantly, when hPRL-G129R was applied together with 4-OH-Tamoxifen, the inhibitory effects were doubled as compared with either the maximum dose of hPRL-G129R or 4-OH-Tamoxifen (Fig. 5). For example, 100 nM of 4-OH-Tamoxifen resulted in an 85% inhibition; yet in the presence of 150 ng/ml of hPRL-G129R, the inhibitory effect resulted in ~58% of control. hPRL-G129R was also able to competitively inhibit hPRL-induced cell proliferation. At a 1:1 molar ratio, hPRL-G129R was able to stop the stimulatory effect of hPRL, and at 2:1 molar ratio, it inhibits cell proliferation (Fig. 6).

Coculture Experiments. We found that stable mouse L-cell lines grow at a similar rate as do regular L cells, regardless of producing either hPRL or hPRL-G129R (data not shown) because of the fact that mouse L cells possess nondetectable levels of PRLR (20). We believe that the coculture experimental set-up sustained the presence of biologically active hPRL-G129R, resulting in a maximal response in these breast tumor cells.

T-47D cells, after coculture with L-PRL or L-PRL-G129R cells, demonstrated dose-dependent growth stimulation (with L-PRL) or inhibition (with L-PRL-G129R; Fig. 7). The responses were rather dramatic as compared with conditioned media experiments. We nearly achieved complete inhibition of cell proliferation.

TUNEL Assay

In this report, we have presented data to demonstrate that the hPRLR antagonist, hPRL-G129R, is able to induce apoptosis by DNA fragmentation in multiple human breast cancer cell lines. The hPRL-G129R induces apoptosis in a dose-dependent manner after 24-h treatment (Fig. 8, *A–F*), and the apoptosis is obvious, even at physiological concentration (50 ng/ml; Fig. 8*C*). To demonstrate the specificity of hPRL-G129R to the hPRLR, hPRL or an anti-hPRL antiserum to reverse the apoptosis process and hPRL-G129R were simultaneously used to treat the cells (Fig. 8, *G–I*). As shown in Fig. 8*H*, hPRL is able to competitively reverse the DNA fragmentation induced by hPRL-G129R at a ratio of 4:1 (500 ng/ml of hPRL *versus* 125 ng/ml of hPRL-G129R). The same results were obtained using BT-474 cells (data not shown). The DNA fragmentation in breast cancer cells is apparent even after 2 h of exposure to hPRL-G129R at a concentration of 50 ng/ml (Fig. 9, *A–D*). We also confirmed that hPRL-G129R could induced apoptosis by DNA fragmentation in four hPRLR-positive breast cancer cell lines after 24 h of treatment. (Fig. 10). To demonstrate the specificity of hPRL-G129R, an anti-hPRL antibody titration experiment was also included (Fig. 8*I*). It was shown that the anti-hPRL antibody could completely block the apoptotic effects of hPRL-G129R in T-47D cells after 6 h of preincubation.

DISCUSSION

Human breast cancer is known to be a heterogeneous mixture of cell clones characterized by different biological features. The primary target of endocrine therapy for breast cancer has been E2, by either surgical or pharmacological methods of estrogen deprivation (1, 2). Among the pharmacological methods, the most notable has been the development of tamoxifen. Recently, the National Surgical adjuvant Breast and Bowel Project has reported the results of the Breast Cancer Prevention Trial demonstrating a 49% decrease in the incidence of invasive breast cancer in a large cohort of high risk women as a result of the use of tamoxifen (32, 33). Despite these encouraging results, a fraction of ER-positive tumors escape first- or second-line endocrine treatment because of the initial presence of estrogen-negative clones or the development of drug resistance. It is this complexity that partly explains why tamoxifen is not universally effective, even in ER-rich tumors (2). In addition, any progress in the development of better antiestrogen therapy for breast cancer is unlikely to impact on the treatment of ER-negative tumors. For these reasons, it is our belief that the scope of the search for drugs to treat breast cancer should be expanded to effectively control tumor growth and/or recurrence in all tumors.

Recently, several lines of evidence strongly suggest that hPRL acts as an autocrine/paracrine growth factor contributing to breast cancer development (11, 34, 35). More importantly, it has recently been reported that sex steroid hormones and PRL interact synergistically to control cancerous growth within the mammary gland (28). ER and PRLR were found being coexpressed and cross-regulated in mammary tumor cell lines as well as in primary breast cancers (28). These findings further suggest that the use of antiestrogen therapy in breast cancer may be attacking only half of the synergistic equation, which leaves an opportunity for further improvement of the ultimate therapeutic approach to breast cancer (28). In support to this notion, a combined regimen using an antiestrogen (Tamoxifen), an anti-GH secretion drug (octreotide), and an anti-PRL secretion drug (CV 205-502) has been reported to have significantly better clinical results in metastatic breast cancer patients as compared with tamoxifen therapy alone (36). Although this regimen does not block the autocrine/paracrine action of PRL on breast cancer, inhibition of circulating PRL from the pituitary did seem to have an additive benefit in the treatment of advanced breast cancer. This raises exciting prospects for even better results with complete PRL blockade with an antagonist that acts at the receptor level.

In this study, we report the design and production a hPRL antagonist, hPRL-G129R. We first demonstrated that hPRL and E2 exhibited an additive stimulatory effect in human breast cancer cell proliferation (Fig. 4). We believe that the synergistic effects between hPRL and estrogen reflect the real physiological status because the breast tissue is constantly exposed to both newly synthesized estrogen and hPRL. These results also indicate the possibility of developing new therapeutic regimens, targeting possible tumor stimuli other than the ER. The potential

for additive and therefore improved benefits is significant. We further demonstrated that hPRL-G129R possessed an inhibitory effect on T-47D cell proliferation (Fig. 5). More importantly, when anti-estrogen (4-OH-Tamoxifen) and anti-PRL (hPRL-G129R) agents were applied simultaneously, as we had anticipated, an additive effect was observed. The inhibitory effect on cell proliferation was more than doubled (Fig. 5). We reason that the direct inhibitory effects of hPRL-G129R on T-47D cell proliferation are by competitive inhibition of the hPRL produced by T-47D cells (11). The hPRLR-specific antagonistic effects of hPRL-G129R were further substantiated by an assay that uses combinations of hPRL and hPRL-G129R. It is encouraging to note that even at the ratio of 1:1, hPRL-G129R could stop the T-47D cell proliferation induced by hPRL (Fig. 6).

We speculated that if we could sustain the effects of hPRL-G129R by providing a continuous fresh supply of antagonist, we might obtain even better results than by a single application and prolonged incubation. To address this question, we designed the coculture experiments. When stable L cells that produce hPRL-G129R were cocultured with T-47D cells, much more dramatic inhibitory effects were observed (Fig. 7). The actual concentration of hPRL-G129R at the end of the experiment is approximately the same as the beginning high dose in the conditioned media experiment; yet apparently because these antagonists are produced continuously, the effects are more dramatic.

Apoptosis (programmed cell death) is one of the central physiological mechanisms that regulates the timely and orderly death of cells (37). The biochemical hallmark of apoptosis is internucleosomal DNA cleavage (38–40), and it can be detected by the TUNEL assay or by conventional gel electrophoresis (41). In this report, we have presented data to demonstrate that the hPRLR antagonist, hPRL-G129R, is able to induce apoptosis by DNA fragmentation in multiple human breast cancer cell lines. The hPRL-G129R induces apoptosis in a dose-dependent manner after 24-h treatment (Fig. 8). The DNA fragmentation in breast cancer cells is apparent even after 2 h of exposure to hPRL-G129R at a concentration of 50 ng/ml (Fig. 9). We further demonstrated the specificity of hPRL-G129R by using either hPRL or an anti-hPRL antiserum to reverse the apoptosis process (Fig. 8). The mitogen rescue effect of hPRL is yet another indication that hPRL-G129R induces apoptosis (39). To our surprise, 4-OH-Tamoxifen did not induce apoptosis in the cell lines we tested at concentrations as high as 1 μM, as assayed by the same protocol (data not shown), suggesting that a different mechanism might be involved. It also explains the additive inhibitory effects on cell proliferation when two agents (hPRL-G129R and 4-OH-Tamoxifen) were applied together (Fig. 5).

The mechanism of induction of apoptosis by this hPRLR antagonist needs further experimental elucidation. The mammary gland is one of the few organs that undergoes most of its development in the mature organism. More importantly, the mammary gland undergoes sequential waves of apoptosis during development and involution beginning with each pregnancy and ending with each weaning. We speculate that PRL might serve as one of the major controlling factors that decides whether the breast cells should go into proliferation/differentiation (by producing more PRL) or apoptosis (deprived of PRL) under physiological conditions. In the case of breast cancer, the cancer cells are adapted to using PRL as a major growth factor by producing PRL on their own (as an autocrine/paracrine growth factor), therefore maintaining their proliferative status. Hence, it is conceivable that when we effectively deprived the mitogenic signal of PRL in breast cancer cells by competitive binding of hPRL-G129R to the hPRLR, apoptosis is induced. Whatever the mechanism of hPRL-G129R-induced apoptosis of breast cancer cells, it is clear that the hPRLR antagonist hPRL-G129R has a strong potential to be used as another line of endocrine therapy along with Tamoxifen or by itself in the treatment of breast cancer.

In summary, the appalling death rate from breast cancer is still a major health care problem in the United States. History and biology have taught us that instead of finding a single magic "bullet" for breast cancer or for any tumor, we are more likely to improve the outcome of patients with oncogenic disease if we consider the heterogeneity of the disease and explore alternative and/or combination treatment regimens. We have reported in this paper a new agent to inhibit breast cancer development, hPRL-G129R, which acts as a hPRL antagonist. These results provided strong evidence of the involvement of hPRL in human breast cancer cell proliferation and also offer a novel approach for the treatment of breast cancer. It is our belief that the development of the hPRL antagonist will have a significant impact on effective human breast cancer therapy.

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A Novel Design of Targeted Endocrine and Cytokine Therapy for Breast Cancer[1]

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ABSTRACT

The aim of this study is to combine endocrine therapy [human prolactin (hPRL) antagonist, G129R] and immune therapy [interleukin 2 (IL2)] in the design of a fusion protein, G129R-IL2, to treat human breast cancer. This novel approach uses the specific interaction between the G129R and hPRL receptors (PRLRs), thus directly targeting the fusion protein to the malignant breast tissues that have previously been shown to contain high levels of PRLR. The localized bifunctional fusion protein is designed to block signal transduction induced by hPRL as well as to activate T lymphocytes near the tumor site. A bacterial expression system was used to produce G129R-IL2 fusion protein that maintained both G129R and IL2 activities as demonstrated by cell-based assays such as signal transducer(s) and activator(s) of transcription (STAT)5 phosphorylation, breast cancer cell proliferation, and T-cell proliferation. The antitumor activities of G129R-IL2 were demonstrated *in vivo* using a syngeneic model system with BALB/c mice and EMT6-hPRLR breast cancer cells. After daily injection (i.p.) of G129R-IL2 (100 μg/mouse) for 18 days, the tumor growth in the G129R-IL2-treated group was only one-third the size as compared with that of the control group. The growth rate in the G129R-IL2-treated group is also significantly slower than that of the group treated with G129R alone (200 μg/mouse/day). We hope that this novel bifunctional protein will contribute significantly to human breast cancer therapy.

INTRODUCTION

One of the leading causes of cancer death in women is metastatic breast cancer. The etiology of breast cancer is complex, but its rarity among males suggests a role of female sex hormones (1, 2). In addition to estrogen, more and more evidence supports the notion that hPRL[3] is also intimately involved in breast cancer development (3–7). The following lines of evidence demonstrate the relationship between PRL and breast cancer: (a) PRL is synthesized by human breast cancer cells, which suggests its autocrine/paracrine role in the mammary gland (4); (b) PRLRs are up-regulated in the majority of malignant breast tissue (8); (c) PRL transgenic mice have high breast cancer rate (9); and (d) the inhibition of the binding of PRL to PRLR inhibits breast cancer cell growth (10).

hPRL is a single-chain, neuroendocrine, polypeptide hormone with 199 amino acids in its mature form. As a member of the GH family, PRL is primarily produced by the lactotrophs of the anterior pituitary gland in all vertebrates. The biological activities of PRL are mediated through specific membrane receptors known as PRLRs. The primary site of PRL action is the mammary gland. In this organ, PRL plays a decisive role in the stimulation of DNA synthesis, epithelial cell proliferation, and the promotion of milk production (11–15). The generation of PRL and *PRLR* gene knockout mice has unambiguously demonstrated that PRL and PRLR are key regulators in mammary development (12, 16).

In previous studies, Chen *et al.* (17–22) have developed a hGH antagonist by making a single amino acid substitution mutation at position 120 of the hGH molecule (hGH-G120R). The mutated hGH has been shown to block GH action both *in vitro* and *in vivo* (21) and has completed its Phase III clinical studies (23). By adopting a strategy similar to the development of the hGH antagonist, Goffin *et al.* (24) and our laboratory (10, 25–27) demonstrated that a single amino acid substitution mutation at position 129 of hPRL resulted in a hPRLR-specific antagonist (G129R). We have demonstrated that G129R is able to inhibit human breast cancer cell proliferation via the induction of apoptosis (10). G129R has also been shown to inhibit tyrosine phosphorylation of oncogene *STAT3* (26, 28) and to modulate transforming growth factor α/β levels in breast cancer cells (27). Furthermore, additive effects of hPRL-G129R and tamoxifen, which serves as an antiestrogen agent, have been

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[3] The abbreviations used are: PRL, prolactin; hPRL, human PRL; CSS, charcoal-stripped serum; FBS, fetal bovine serum; GH, growth hormone; hGH, human GH; IL2, interleukin 2; hIL2, human IL2; PRLR, PRL receptor; IRMA, immunoradiometric assay; mAb, monoclonal antibody; STAT, signal transducer(s) and activator(s) of transcription; ATCC, American Type Culture Collection; RT-PCR, reverse transcription-PCR; TBS, Tris-buffered saline; MTS-PMS, (3-4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium, inner salt, phenazine methosulfate.

observed (10). Taken together, the ability of G129R to inhibit breast cancer cell proliferation, especially its additive effects with tamoxifen, makes it potentially valuable as a therapeutic agent for the treatment as well as prevention of breast cancer.

Tumor immune therapy has been of great interest for many years (29, 30). IL2 has been one of the main cytokines used for treating cancer. IL2, originally called T-cell growth factor, is a M_r 15,000 glycoprotein encoded by a single gene on chromosome 4 in humans (31). Characteristics of IL2 that make it attractive in cancer therapy include its ability to stimulate T lymphocytes as well as natural killer cells (32). However, one of the disadvantages in using IL2 is that patients systemically receiving IL2 often experience serious side effects that limit the amount of IL2 that can be administered. This limitation of dosage in turn directly affects the efficacy of treatment (33, 34).

In this study, we explore the possibility of fusing G129R with IL2 in the hope of generating a bifunctional protein that will have a dual therapeutic effect (targeted endocrine and cytokine) in the treatment of breast cancer. The targeting ability of this novel fusion protein uses the highly specific interactions between ligand (G129R) and receptor (PRLR). After G129R binds to PRLR, it not only blocks the signal transduction inducted by PRL but also localizes IL2 at the tumor site, which will play a crucial role in T lymphocyte activation, thus leading to tumor cytotoxicity. Because IL2 would be concentrated mainly in the breast cancer tissue, the severe side effects of IL2 would be greatly reduced.

MATERIALS AND METHODS

Cell Lines and Animals. To test the dual function of the fusion protein, human breast cancer cells (T-47D) and T cells (HT-2) were used for *in vitro* studies. T-47D human breast cancer cells were purchased from ATCC (Manassas, VA) and maintained in RPMI 1640 (Life Technologies, Inc. Inc., Rockville, MD) supplemented with 10% FBS and 50 μg/ml gentamicin. The HT-2 cell line, a murine T cell line requiring IL2 for growth, was also obtained from ATCC and cultured in RPMI 1640 containing 10% FBS, 200 IU of IL2 (kindly provided by Dr. Samuel Smith, Greenville Hospital System, SC), and other ATCC-recommended supplements. In addition, because of the nature of the fusion protein, a syngeneic animal tumor model (tumor cells paired with a immunocompetent host of identical genetic background) must be chosen as a study model. The EMT6 mouse mammary tumor cells, which originated from BALB/c mouse mammary carcinoma, were kindly provided by Dr. Rockwell, Yale University (New Haven, CT). After initial examination by RT-PCR, we found that the expression level of PRLR was nondetectable in these cells; therefore, a subline of EMT6 cells was generated in which full-length hPRLR cDNA was stably transfected using G418 selection as described previously (35). In a separate experiment, primers specific for mouse IL2 receptor were used to detect the IL-2 receptor expression. The results were negative (data not shown). The EMT6-hPRLR cells were maintained in DMEM (Life Technologies, Inc.) supplemented with 10% FBS (Life Technologies, Inc.) and 50 μg/ml gentamicin (Life Technologies, Inc.). All of the cell lines were grown at 37°C in a humid atmosphere in the presence of 5% CO_2.

The animals used for this study were 8–10-week-old female BALB/c mice (Jackson Laboratory; Bar Harbor, ME), which were housed in compliance with NIH guidelines.

Cloning of G129R-IL2 Fusion cDNA for *Escherichia coli* Expression. A two-step cloning procedure was used to generate a recombinant DNA encoding G129R fused to IL2. Primers corresponding to G129R (minus sequences encoding signal peptide and stop codon, and plus restriction sites of *Nde*I and *Bam*HI: 5′-*CAT ATG* TTG CCC ATC TGT CCC GGC-3′ and 5′-*GGA TCC* GCA GTT GTT GTT GTG GAT-3′) were used to amplify the G129R fragment from pCR3.1-G129R (10). Primers corresponding to hIL2 (minus sequences encoding signal peptide, and plus restriction sites of *Bam*HI and *Xho*I: 5′-*GGA TCC* GCA CCT ACT TCA AGT TCG-3′ and 5′-*CTC GAG* TTA AGT TAG TGT TGA GAT GAT-3′) were used to amplify the hIL2 fragment from hIL2 cDNA, purchased from ATCC. Both fragments were cloned into pCR2.1 TA cloning vector (Invitrogen, Inc., Carlsbad, CA) and sequenced. The fragments were reisolated by restriction digestion, purified, and ligated into the pET22b+ expression vector (Novagen, Madison, WI; Fig. 1).

Production and Purification of G129R-IL2. BL21 (DE3) cells (Novagen) were transformed with pET22b-G129R-IL2 using the calcium chloride method. An *E. coli* BL21 (DE3) seed culture (200 ml) carrying the pET22b-G129R-IL2 plasmid was grown overnight at 37°C and was used to inoculate 4 liters of L-broth (Bio 101, Carlsbad, CA) containing 100 μg/ml ampicillin (Fisher Scientific, Fair Lawn, NJ). The culture was grown at 37°C with agitation until the $A_{600\ nm}$ reached 0.9, at which time 1 mM isopropyl β-thiogalactoside (IPTG; Alexis Biochemicals, San Diego, CA) was added to induce expression of T7 RNA polymerase; the culture was incubated for an additional 3 h. The cells were then harvested by centrifugation at 6,000 × *g* for 5 min and resuspended in 0.2 M $NaPO_4$ (pH 8), 10 mM EDTA, 0.1 mg/ml lysozyme, and 0.5% Triton X-100, and incubated at 37°C for 1 h. The cells were disrupted by sonication using five 1-min pulses at 5 kHz applied with a Vibra-Cell Sonicator (Fisher Scientific). The insoluble inclusion bodies were recovered by centrifugation at 12,000 × *g* for 15 min at 4°C; resuspended in 0.2 M $NaPO_4$ (pH 7), 5 mM EDTA, 1 M urea, and 0.5% Triton X-100; recollected by centrifugation at 12,000 × *g* for 15 min; resuspended in 0.2 M $NaPO_4$ (pH 8), 8 M urea, and 1% v/v β-mercaptoethanol; and heated at 55°C for 10 min. Renaturation of the solubilized G129R-IL2 was performed by dialysis against decreasing concentrations of urea/TE buffer [20 mM Tris, 2 mM EDTA (pH 8.3)] for 4 days. The renatured protein was then filtered with 0.45 μm filters and purified using an anionic exchange column (Q-Sepharose) on a fast-performance liquid chromatography system (Amersham Pharmacia, Newark, NJ). The concentration of G129R-IL2 was determined using a hPRL IRMA kit (DPC, Inc., Los Angeles, CA), and its purity was determined via silver staining using the Silver Stain Plus kit (Bio-Rad Inc., Hercules, CA).

Verification of Fusion Protein Production via Western Analysis. Samples (200 ng) were analyzed using 4–15% SDS PAGE followed by Western blotting. After SDS-PAGE, the protein was transferred to ECL Hybond nitrocellulose (Amersham Pharmacia) at 16 W for 1.5 h. Blots were blocked with TBS containing 5% milk and 0.05% Tween 20 (blocking buffer)

for 30 min at room temperature; incubated overnight at 4°C in blocking buffer containing the appropriate antibody [IL2 antiserum, 1:200 (Santa Cruz Biotechnology Co., Santa Cruz, CA); hPRL antiserum, 1:1000 (Dr. Parlow, National Hormone & Pituitary Program, NIH, Bethesda, MD)]. The blots were then washed three times with TBS containing 0.05% Tween 20 (5 min/wash); and incubated in goat antirabbit secondary antibody (1:5000; Bio-Rad, Hercules, CA) for 1.5–2 h at room temperature with constant agitation. After secondary antibody incubation, membranes were washed three times with TBS-Tween-20 (5 min/wash); developed for 1 min using enhanced chemiluminescence reagents (Amersham Pharmacia) and captured on Kodak MR film (Fisher Scientific).

STAT5 Assay. Twenty-four h before protein extraction, T-47D cells were grown to confluency in 6-well plates containing RPMI 1640 supplemented with 10% CSS (Hyclone, Logan, UT). On the day of treatment, T-47D cells were depleted for 30 min in RPMI 1640 containing 0.5% CSS. The cells were treated for 20 min with the appropriate amount of hPRL (Dr. Parlow, National Hormone and Pituitary Program, NIH), G129R, or G129R-IL2, washed with ice-cold PBS (Life Technologies, Inc.), lysed with 200 μl of lysis buffer [50 mM Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM Na_3VO_4] and incubated on an orbital rotator for 15 min. The lysate was transferred to 1.5-ml centrifuge tubes, gently passed through a 21-gauge needle five to six times to shear genomic DNA, and then placed on ice for 20 min. The lysate was centrifuged at 12,000 × *g* for 20 min at 4°C, and the supernatant was removed and stored at −20°C until ready for use.

Thirty-five μl of cell lysate (65–70 μg) was used for Western blotting analysis as described in the previous section using STAT5A + STAT5B antiserum (1:4000 dilution; UBI; Lake Placid, NY) or with anti-phospho-STAT5 antiserum (UBI) at a concentration of 1.5 μg/ml (26).

Radioreceptor Binding Assay. PRL receptor binding assays were performed on EMT6-hPRLR cells using T-47D human breast cancer cells as well EMT6 parental cells as controls, as described previously (10). Briefly, cells were grown in six-well tissue-culture plates until 90% confluent (~1×10^5 cells/well). Monolayers of cells were starved in serum-free RPMI 1640 for 0.5–1 h. The cells were then incubated at room temperature in serum-free RPMI 1640 containing 5×10^4 cpm ^{125}I- hPRL (specific activity, 30 μCi/μg; NEN Perkin-Elmer, Boston, MA) with or without 500 ng/ml hPRL. Cells were washed three times in serum-free RPMI 1640, lysed in 0.5 ml of 0.1 N NaOH/1%SDS, and the bound radioactivity was determined by scintillation counting. Total specific binding was calculated and compared.

EMT6-hPRLR Breast Cancer Cell Proliferation Assays. The assay conditions were modified from those described by Ginsburg and Vonderhaar (4). EMT6 cells were trypsinized and transferred to 96-well plates containing DMEM supplemented with 1% CSS. The optimal cell number/well for EMT6 cells was found to be 15,000 cells/well using titration assays. The cells were allowed to settle and adhere overnight (12–18 h), and various concentrations of hPRL, G129R, or G129R-IL2 were added. The cells were incubated for an additional 24 h at 37°C in a humidified 5% CO_2 incubator. After incubation, MTS-PMS solution (Cell Titer 96 Aqueous kit; Promega Corp., Madison, WI) was added to each well following the manufacturer's instructions, and the plates were read at 490 nm using a BIO-RAD benchmark microplate reader (Hercules, CA). All of the experiments were carried out in triplicate.

HT-2 Cell Proliferation Assay. Before each assay, HT-2 cells were washed three times in growth medium lacking IL2. The cells were counted, and ~5×10^3 cells were transferred to each well of a 96-well plate. Dose-response curves were obtained by varying the concentration of IL2, G129R-IL2, PRL, or G129R added to the HT-2 cells and incubating for 24 h at 37°C. Cell proliferation assays (MTS-PMS; Promega) were performed in triplicate using the same procedure described in the previous section.

***In Vivo* Studies of Antitumor Efficacy of G129R-IL2.** Two experiments were conducted to determine the antitumor efficacy of G129R-IL2 fusion protein using EMT6-hPRLR cells and a BALB/c female mouse model. In the first experiment, eight eight-week-old female BALB/c mice were inoculated s.c. with 1×10^6 EMT6-hPRLR breast cancer cells and randomized into two groups. One day after breast cancer cell inoculation, mice were injected (i.p.) with either 50 μg/mouse of G129R-IL2 or PBS every 24 h for 14 consecutive days. In the second experiment, 24 mice were given s.c. injections of 1×10^6 EMT6-hPRLR breast cancer cells and were randomized into four groups. One day after breast cancer cell inoculation, mice were given injections (i.p.) of PBS, G129R (200 μg/mouse), or G129R-IL2 (100 μg/mouse or 200 μg/mouse, respectively) for 18 consecutive days. At the end of the experiments, the tumors were dissected and weighed. It should be pointed out that the original experimental design included a group of animals that was treated with 20 μg of free IL2 mixed with 20 μg of G129R/mouse. Because of the toxicity of IL2 to the mice, the mice died and the experiment ended. The data are expressed as mean ± SE, and the Student *t* test was used to analyze the statistical difference between groups.

RESULTS

Construction of pET22b-G129R-IL2 Expression Vector. G129R-IL2 cDNA was cloned into the pET22b(+) expression vector as shown in Fig. 1. The G129R and IL2 cDNA sequences were found to be identical to those reported in GenBank, except for a single codon mutation (GGC to CGG), which resulted in Gly to Arg mutation at position 129 of hPRL (accession no. XM 033558). Two amino acids, Gly and Ser, were added at the junction of G129R and IL2 because of the addition of a *Bam*HI (GGATCC) restriction site for cloning purposes.

Production of G129R-IL2 Fusion Protein. The G129R-IL2 fusion protein was produced in the form of inclusion bodies. After refolding and ionic exchange column purification, the yield of fusion protein was ~2 mg/liter as determined by the Bradford protein assay and PRL IRMA analysis. The purified protein was analyzed by 4–15% SDS PAGE followed by silver staining (Fig. 2*A*), and the identity of the M_r 38,000 fusion protein was further confirmed by Western analysis using antiserum against hIL2 (accession no. XM 035511) or hPRL, respectively (Fig. 2*B* and 2*C*).

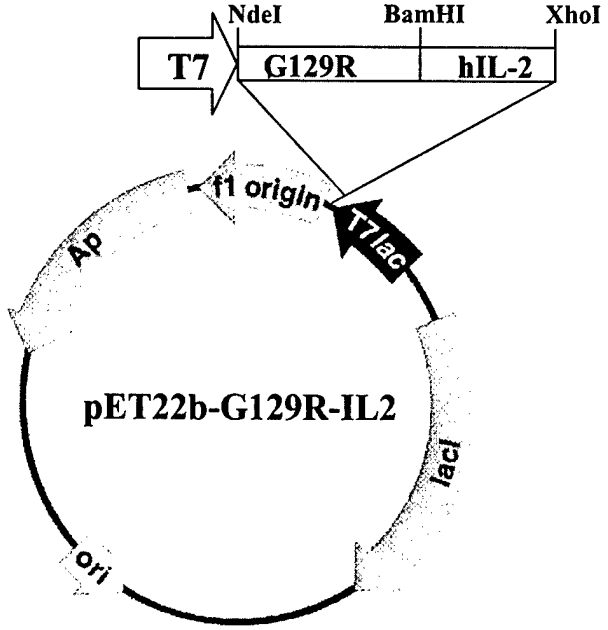

Fig. 1 Cloning and construction of the expression plasmid for G129R-IL2 production. PCR fragments amplified from G129R and hIL2 cDNAs were ligated into an *E. coli* expression vector, pET22b+, resulting in pET22b-G129R-IL2. A *Bam*HI restriction site was created between G129R and IL2 cDNAs for cloning purposes. The addition of the *Bam*HI site resulted in two extra amino acid residues (Gly and Ser).

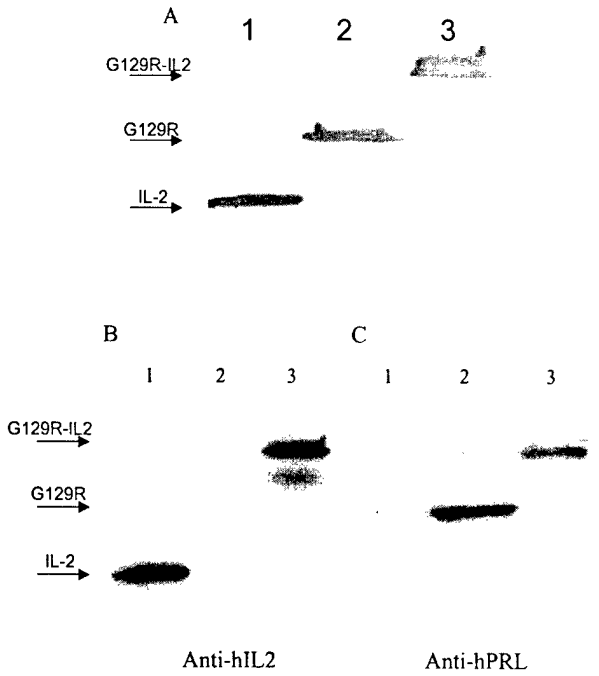

Fig. 2 Production and purification of G129R-IL2. *A*, silver staining of a SDS-PAGE gel. IL2 (*Lane 1*), G129R (*Lane 2*), or G129R-IL2 (*Lane 3*) were analyzed by 4–15% SDS-PAGE followed by silver staining (200 ng/lane). *B*, and *C*, Western blot analyses. IL2 (*Lane 1*), G129R (*Lane 2*), or G129R-IL2 (*Lane 3*) were analyzed by 4–15% SDS-PAGE (100 ng/lane) followed by Western blotting with either IL2 antiserum (*B*) or hPRL antiserum (*C*).

HT-2 Cell Proliferation Assay. An HT-2 cell proliferation assay was used to determine whether or not the IL2 portion of the fusion protein was functional. Fig. 3*A* demonstrated a dose response of IL2 in the proliferation of HT-2. The stimulatory effect of G129R-IL2 fusion protein on HT-2 cell proliferation was similar to that caused by IL2 alone (Fig. 3*B*). G129R or hPRL alone had no effect on HT-2 cell proliferation (Fig. 3*C*). The EC_{50} values for the G129R-IL2 and IL2 were ~1 ng/ml.

STAT Assay. Fig. 4*A* demonstrates a dose response of STAT5 phosphorylation in T-47D cells induced by hPRL. STAT5 phosphorylation was detected at a maximum level for 100 ng/ml hPRL (Fig. 4*A*). G129R (Fig. 4*B*) and IL2 (Fig. 4*C*), on the other hand, were inactive in this assay. To determine the antagonistic effects of G129R-IL2, T-47D cells were treated with a constant concentration of hPRL (100 ng/ml) and various concentrations of G129R or G129R-IL2 fusion protein (50 ng/ml to 1 μg/ml). It can be seen that at a 1:5 ratio (hPRL: G129R), STAT5 tyrosine phosphorylation is significantly decreased (Fig. 5*A*); and at a 1:10 ratio (hPRL:G129R), STAT5 tyrosine phosphorylation is almost completely inhibited (Fig. 5*A*). Fig. 5*B* demonstrates that G129R-IL2 fusion protein inhibits STAT5 phosphorylation induced by hPRL to nearly the same extent as G129R; therefore, the G129R portion of the fusion protein is functional.

Generation of EMT6-hPRLR Cells. The tumor cell line used for the *in vivo* studies was the EMT6 mouse mammary tumor cell line. Because this cell line has nondetectable PRLR mRNA as determined by RT-PCR (Fig. 6*A*, *Lane 2*), EMT6 cells were transfected with hPRLR cDNA to generate an EMT6-hPRLR stable cell line. Fig. 6*A* (*Lane 4*) shows the results of RT-PCR that demonstrate the expression of hPRLR mRNA in the EMT6-hPRLR cell lines. The hPRLR mRNA expression level in the EMT6-hPRLR cell line selected was still found to be much lower than that of T-47D cells (Fig. 6*A*).

The hPRL receptor status in EMT6-hPRLR cells was confirmed by using a radio receptor assay. The results of a direct comparison of the hPRL receptor-specific binding levels in the three breast cancer cell lines are shown in Fig. 6*B*. T-47D cells have the highest specific PRL receptor binding and EMT6 parental cells have close to zero binding. The EMT6-hPRLR cells demonstrate ~12% of specific binding. The results of receptor binding correlate well with the RT-PCR data.

Once the EMT6-hPRLR cell line was established, the effects of hPRL, G129R, and G129R-IL2 on cell proliferation of this cell line were investigated. When equal numbers of cells (15,000) were treated with 500 ng/ml hPRL, G129R, or G129R-IL2, the stimulatory effects were seen from only the cells treated with hPRL; whereas both G129R and G129R-IL2 demonstrated inhibitory effects on EMT6-hPRLR cell proliferation (Fig. 7*A*). More importantly, G129R or G129R-IL2 (1:10) competitively inhibited the proliferative effects induced by hPRL (Fig. 7*B*).

***In Vivo* Studies of the G129R-IL2 Fusion Protein.** Pharmacokinetic studies of G129R-IL2 were first conducted to determine the effective dose needed. Eight-week-old female BALB/c mice were given injections i.p. of either 25 μg/mouse

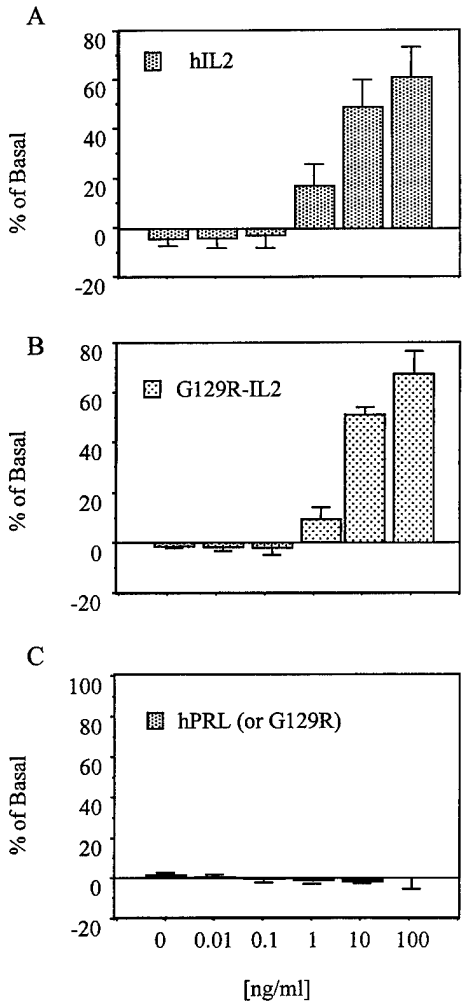

Fig. 3 HT-2 cell proliferation assay in response to IL2, G129R, or G129R-IL2. *A*, dose-response effects of HT-2 cells on hIL2. *B*, dose-response effects of HT-2 cells on G129R-IL2; *C*, HT-2 cell incubated with either hPRL or G129R.

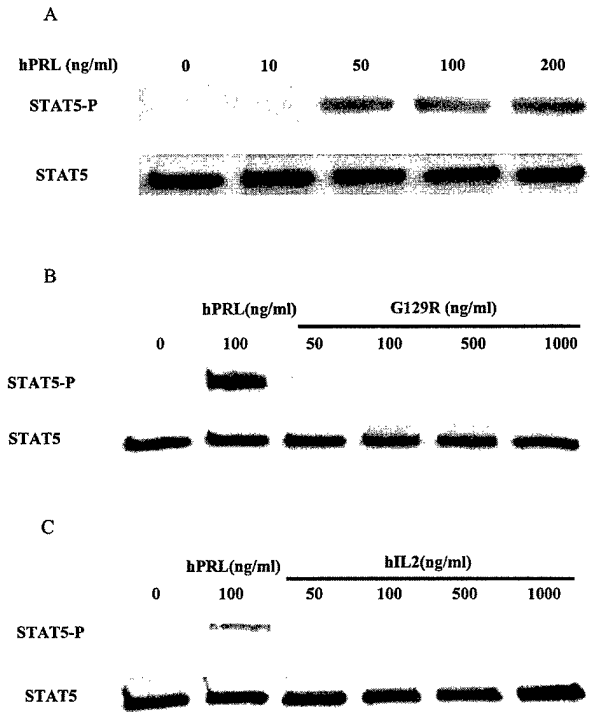

Fig. 4 Stimulation of STAT5 phosphorylation by hPRL. T-47D human breast cancer cells were treated with the indicated concentrations of hPRL, G129R, or IL2. Total protein was extracted from treated cells and analyzed via 4–15% gradient SDS-PAGE, followed by Western blotting with antiserums against either STAT5 or Phospho-STAT5 as indicated in each panel. *A*, dose-response effects of hPRL on STAT5 phosphorylation; G129R (*B*) and IL2 (*C*) are inactive in this assay.

(n = 4) or 50 μg/mouse (n = 3) of the G129R-IL2 fusion protein. After injection, serum samples were collected via tail vein bleeding at 2, 6, and 24 h. The concentration of the fusion protein was assayed by the hPRL IRMA kit (DPC, Inc.). Fig. 8 shows that the serum G129R-IL2 concentration 24 h after injection was ~20 ng/ml This finding is somewhat surprising because hPRL or G129R had a serum half-life of ~2 h and the half-life of IL2 was even shorter. We normally could not detect G129R 24 h after injection. With these encouraging results, it was decided that mice that bore mammary tumor cells would be treated every 24 h.

In our preliminary animal studies, eight female BALB/c mice were inoculated s.c. with 1×10^6 EMT6-hPRLR breast cancer cells and randomized into two groups. Each animal then received daily injections of G129R-IL2 (50 μg/mouse). We found that the serum concentration of fusion protein was maintained at ~30 ng/ml, which reduced the growth of EMT-6-PRLR in mice (115 ± 55 mm^3 *versus* 238 ± 75 mm^3 in control group). Although because of small sample numbers, no statistical difference could be found in tumor volume nor in final tumor weights between the two groups, it provided dose reference for our main animal studies.

Twenty-four female BALB/c mice were inoculated s.c. with 1×10^6 EMT6-hPRLR breast cancer cells and randomized into four groups. Fig. 10 demonstrates that the tumor growth was similar between the groups treated with G129R (241 ± 45 mm^3; 200 μg/day/mouse) and a high dose of G129R-IL2 (223 ± 41 mm^3; 200 μg/day/mouse); however, mice that were given injections of 100 μg of G129R-IL2 showed the best response, in which the average tumor volume was approximately one-third of that in the control group (125 ± 25 mm^3 *versus* 305 ± 55 mm^3).

DISCUSSION

Recent advances in the understanding of the immune system and in defining tumor antigens have motivated the development of many new strategies using immune therapy in cancer treatment (36–38). There is ample evidence that cancers express tumor-specific antigens and that hosts have T cells that can respond to these antigens (39, 40). However, it is likely that tumor cells are poor antigen-presenting cells because they do not provide second signals, which are needed for full T-cell activation (40). Therefore, the major effort in tumor immune

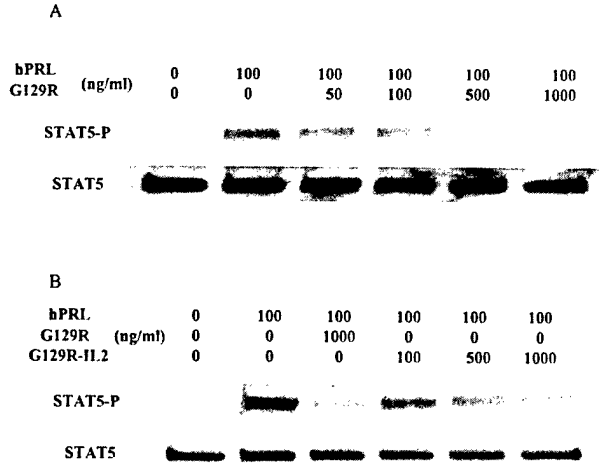

Fig. 5 Inhibition of STAT5 phosphorylation by G129R or G129R-IL2 in T-47D human breast cancer cells. T-47D cells were treated with the indicated concentrations of hPRL, G129R, and G129R-IL2 or with combination as indicated. Total protein was extracted from cells and analyzed via 4–15% gradient SDS-PAGE followed by Western blotting with antiserums against either STAT5 or Phospho-STAT5 as indicated in each panel. *A*, the competitive inhibition of STAT5 phosphorylation by G129R. *B*, the competitive inhibition of STAT5 phosphorylation by G129R-IL2.

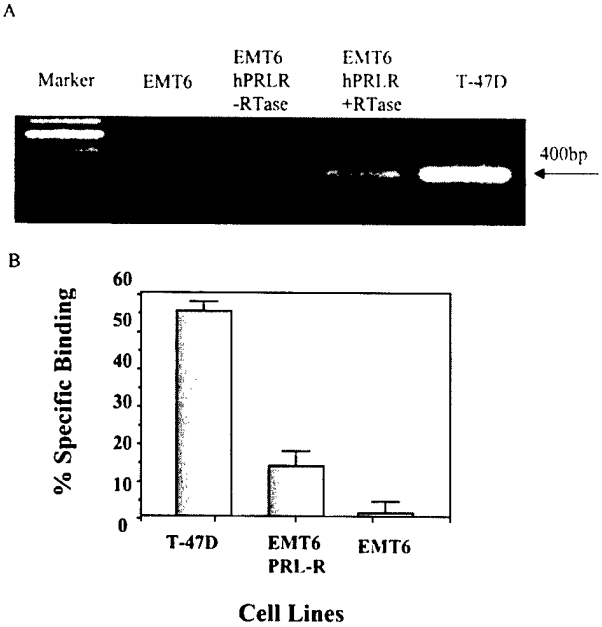

Fig. 6 Confirmation of the expression of hPRLR in EMT-6-hPRLR cells. *A*, RT-PCR analysis of hPRLR mRNA level using total RNA isolated from EMT6 or EMT6-hPRLR cells. RT-PCR products were analyzed on a 1% agarose gel as indicated. *Arrow*, a 400-bp fragment. *B*, results of radioreceptor binding assay on three breast cancer cell lines. Specific binding of PRL recepter was measured using the formula: [(cpm of total binding − cpm of nonspecific binding)/cpm of total binding] × 100.

therapy is focused on how to augment weak host immune responses to tumor antigens, such as exogenously administering cytokines to the patients. Among the many cytokines used, IL2 has been demonstrated to yield promising results (36–38).

IL2 is the principal cytokine responsible for the progression of T lymphocytes from the G_1 to S phase of the cell cycle. It is mainly produced by CD4+ T cells and in smaller quantity by CD8+ T cells (41). With the help of recombinant DNA technology, recombinant hIL2 has been used *in vivo* to treat patients with advanced renal cell carcinoma and melanoma (33, 34). The aim of such an approach is to generate tumor-reactive lymphocytes in cancer patients. However, it has been reported that cancer patients receiving systemic hIL2 often experience potentially life-threatening side effects that limit the total amount that can be administered, which in turn directly affects the efficiency of treatment (33, 34). The major efforts regarding the use of IL2 in tumor therapy, therefore, have been concentrated on how to balance the side effects and the effective dose. By increasing the specificity of administered IL2 (via the targeting of IL2 precisely to the tumor sites), it is possible to dramatically increase the therapeutic effects of hIL2 while significantly decreasing its side effects.

Recently an alternative approach for using the binding specificity of antitumor mAbs to direct cytokines to tumor sites has been introduced (40–46). This novel approach combines the unique targeting ability of mAbs with the activities of cytokines and, therefore, achieves an effective concentration of IL2 in the tumor microenvironment. The targeted IL2 therapy has been shown to be able to completely eradicate disseminated pulmonary and hepatic murine melanoma metastases in immunocompetent syngeneic mice (42, 43) and has also generated promising clinical results (47). These findings demonstrate that targeted IL2 can provide an effective tool in cancer immunotherapy and establish the missing link between T-cell-mediated cytotoxicity and objective clinical response. There are several obvious advantages of this targeted IL2 therapy. First, an mAb-IL2 fusion protein does not have to reach all of the target cells to achieve the maximum effects because it is not a direct cytotoxic reaction (46, 48). Second, it has been shown that the induction of a cellular immune response using the mAb-targeted IL2 approach facilitates the eradication of established s.c. melanoma metastases, even if the tumor displays substantial antigen heterogeneity (47). Most importantly, the therapeutic effect of targeted IL2 therapy is associated with the induction of a long-lived and transferable, protective tumor immunity. In addition, this mAb-targeted IL2 therapy is also different from, and advantageous to, the *ex vivo* transfer of cytokine genes because it concentrates IL2 in the tumor environment in a nonpersonalized way that makes this approach clinically more feasible (42–50).

In our previous studies, we have demonstrated that G129R was able to inhibit breast cancer cell proliferation via the induction of apoptosis both *in vitro* (10, 26–27) and *in vivo* (25). In this study, we used a strategy similar to that of mAb-IL2 to design a novel G129R-IL2 fusion protein that is targeted specifically to human breast cancer. The targeting ability of this novel fusion protein involves the highly specific interactions between the ligand (G129R) and receptor (PRLR), therefore, concentrating IL2 at the cancerous breast tissue in which PRLR levels have been shown to be elevated (8). We hypothesized that once the G129R-IL2 fusion protein reaches the malignant mam-

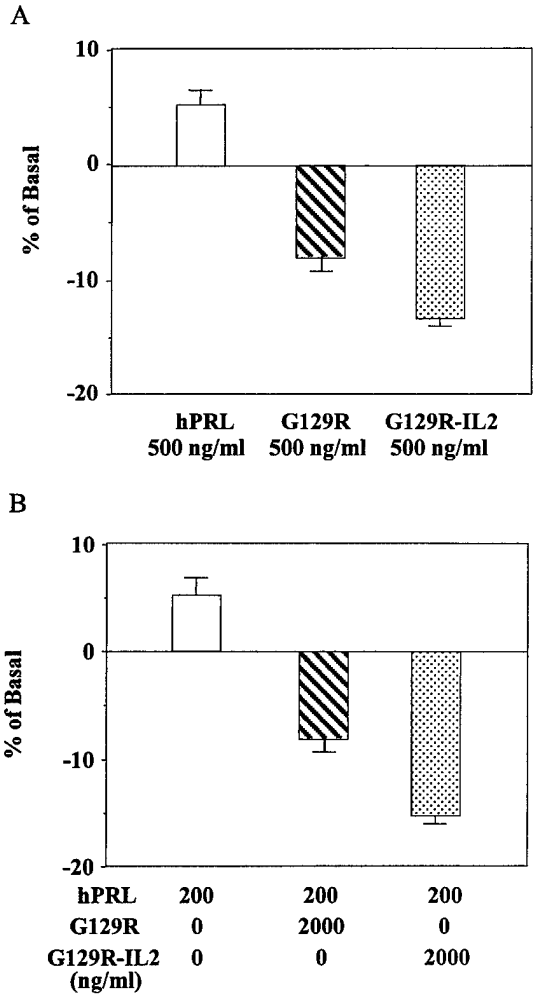

Fig. 7 Inhibition of EMT6-hPRLR cell proliferation by G129R or G129R-IL2. EMT6-hPRLR cells were treated with hPRL, G129R, G129R-IL2, or in combination as indicated. In *A*, PRL induces cell proliferation of EMT6-hPRLR cells, whereas both G129R and G129R-IL2 have inhibitory effect on the proliferation of EMT6-hPRLR cells. In *B*, G129R or G129R-IL2 is able to competitively inhibit the stimulatory effect of hPRL on EMT6-hPRLR cells. The inhibitory effect of G129R-IL2 is significantly greater than that of G129R ($P < 0.05$).

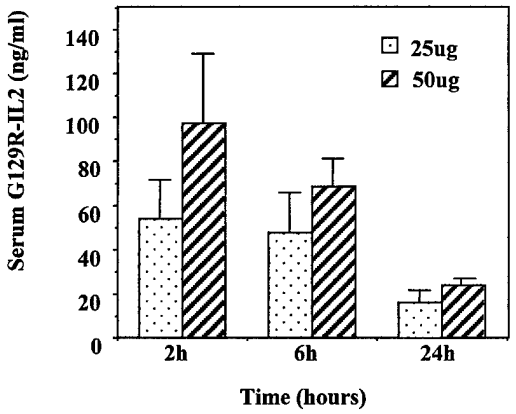

Fig. 8 Pharmacokinetic studies of G129R-IL2 in Balc/c mice. BALB/c mice were given injections (i.p.) of either 25 μg or 50 μg of G129R-IL2, and serum samples were collected via tail vein bleeding at time intervals indicated. The serum concentration of G129R-IL2 was determined via the hPRL IRMA kit.

mary tissues, it will elicit dual therapeutic effects: the G129R portion of the fusion protein will specifically block PRLR, inhibiting the autocrine/paracrine effects of endogenous PRL; and the IL2 portion of the fusion protein may elicit a T-cell-mediated antitumor cytotoxicity reaction *in situ*, as in the case of mAb-IL2 studies.

To express the G129R-IL2 fusion protein, several different cloning strategies were used. Eukaryotic expression systems were not effective and resulted in very low yields, which made it impractical for *in vivo* studies. Ultimately, the bacterial expression vector pET22b+ was used to produce relatively large quantities of the G129R-IL2 fusion proteins. although the yield was far from ideal when compared with the yield of G129R. The low yield of production may be, in part, attributable to the presence of five pairs of Cys residues (three pairs in hPRL and two pairs in hIL2) in this novel protein. Only a small portion of the protein was found to be able to refold properly and eluted from Q-Sepharose columns in low-salt fractions (0.15 mM NaCl). G129R-IL2 fusion protein in these fractions is fully active in cell-based assays. More than 60% of the fusion protein eluted from the Q-Sepharose columns in high-salt fractions (>1 M NaCl) was nonfunctional as tested by STAT5 and HT-2 assay. We believe that proteins in the high-salt fractions represent fusion protein with mismatched disulfur bonding, which results in nonfunctional conformations.

The HT-2 proliferation analysis and STAT assays indicated that properly refolded G129R-IL2 fusion protein retained its IL2-like activity, namely stimulation of T-cell proliferation, as well as G129R-like activities, namely inhibition of STAT5 phosphorylation and inhibition of breast cancer cell proliferation. Although the fusion protein was functional *in vitro*, the real challenge was to determine whether this fusion protein could function *in vivo*. Pharmacokinetic results indicated that the blood clearance of G129R-IL2 fusion protein is much slower than that of either G129R alone or IL2 alone. The serum concentration of G129R-IL2 remained at 20–30 ng/ml after daily injection (50 μg/mouse/day). These data are very significant because previous studies have shown that the half-life of G129R or hIL2 are less than 2 h because of small molecular sizes. Moreover, the serum concentration of G129R or IL2 was not detectable 24 h after injection with a dose up to 200 μg/mouse. We believe that the significantly prolonged serum half-life of G129R-IL2 could not be explained merely by the increase in size of the fusion protein. It was reported that IL2 is able to bind to α-macroglobulin in serum (51), therefore, prolonging its serum half-life. This unique feature of IL2 might help to prolong the half-life of the G129R-IL2 fusion protein.

The concentration of G129R-IL2 used in our *in vivo* studies was similar to the dose used in hGH antagonist clinical studies (5–10 mg/kg of body weight) and is also in the range of G129R used alone in our recent *in vivo* studies with human breast cancer cell xenografts in nude mice (25). It is noteworthy that the concentrations of fusion protein used in our *in vivo* studies

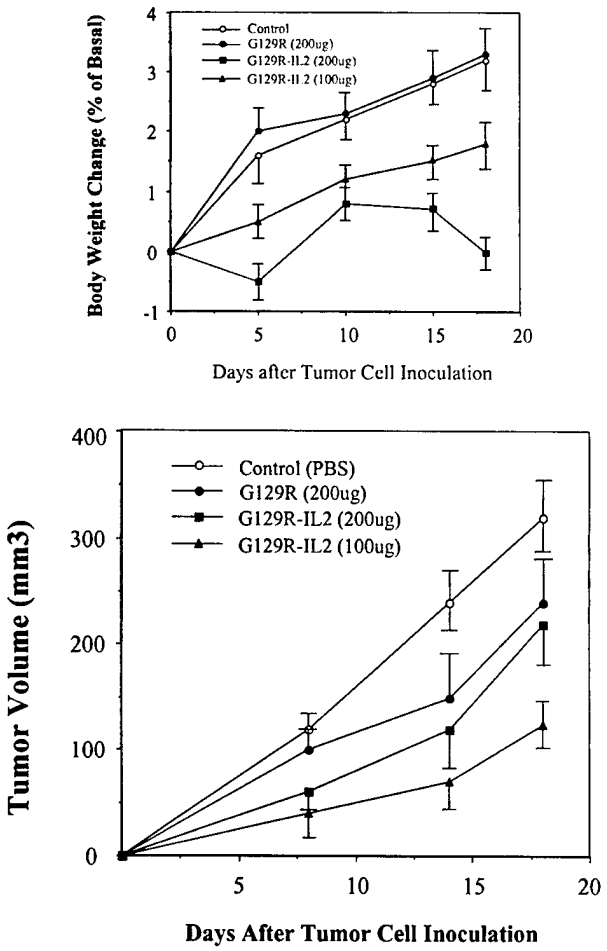

Fig. 9 Inhibition of EMT6-hPRLR cell growth *in vivo* by G129R-IL2. Twenty-four Balb/C mice were given injections of 1×10^6 EMT6-hPRLR cells. After tumor inoculation, mice were randomized into four groups and treated with PBS, G129R (200 μg/mouse/day), or G129R-IL2 (100 μg/mouse/day or 200 μg/mouse/day) for 18 consecutive days. Tumor volumes were calculated by the following equation: [(short dimension^2) × (long dimension)]/2. *Parentheses*, the average body weight of each group.

are considered highly toxic when the molar concentration of its IL2 portion is considered. In our preliminary studies, IL2 (20 μg/mouse/day) i.p. injection has proved very toxic. One animal died 3 days after i.p. injection, the remaining three were extremely ill, and the experiment was terminated. These results strongly suggest that the pharmacodistribution pattern of G129R-IL2 is different from that of free IL2 despite the prolonged serum exposure to the fusion protein. One explanation of the lower toxicity observed in G129R-IL2 fusion protein is that it binds to the PRLR and, therefore, is concentrated quickly in tissues with high levels of PRLR, thus decreasing systemic exposure of IL2. An alternative explanation is that G129R-IL2 acts differently at the receptor level (although G129R-IL2 is able to stimulate HT-2 proliferation) as compared with free IL2, because the molecular size is more than doubled. However, we noticed that the tumor-inhibitory effect of the fusion protein in the group of 100-μg/day/mouse is better than that of the 200-μg/day/mouse group. We believe that the discrepancy between these results is attributable to the toxic reaction caused by the high dose of G129R-IL2 (200 μg/day/mouse). This speculation was supported by the observation that there is a body weight loss during the treatment period in the high-dose group (Fig. 9).

We also directly compared the inhibitory effects of G129R and G129R-IL2 in cancer cell proliferation assay (Fig. 7) as well as the growth of xenografts (Fig. 9). In both cases, G129R-IL2 showed stronger inhibitory effects than G129R alone. We assume that the better *in vivo* results are attributable to the effects of targeted IL2 and prolonged serum half-life of G129R, although additional studies regarding the immune response *in vivo* after the administration of G129R-IL2 are needed. We do not have a good explanation for the difference between G129R and G129R-IL2 in the inhibition of EMT6-hPRLR cell proliferation. We speculate that G129R-IL2 is probably more stable in cultured media as compared with G129R and, therefore, results in better inhibitory effects.

In conclusion, the data presented here demonstrate that the fusion of G129R and IL2 results in a novel, bifunctional protein, G129R-IL2. This novel fusion protein is able to act as a PRLR antagonist as well as a T-cell growth factor. With a relatively long serum half-life, daily injection of G129R-IL2 at a dose of 100 μg/mouse resulted in significant inhibition of breast tumor growth *in vivo*. Additional *in vivo* studies regarding the fusion protein's biological activities using natural breast cancer cells are needed to evaluate its bifunctional properties. We believe that this targeted endocrine-immune design provides a novel and effective approach to human breast cancer treatment.

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Regulation of *bcl-2* gene expression in human breast cancer cells by prolactin and its antagonist, hPRL-G129R

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To gain insight into the molecular basis of human prolactin (hPRL) antagonist induced apoptosis, we compared the differential gene expression profile of four human breast cancer cell lines following treatment with hPRL and its antagonist (hPRL-G129R). Among the genes identified, the *bcl-2* gene was of particular interest. We found that *bcl-2* mRNA was up regulated in three of the four cell lines that were treated with hPRL. To further confirm these results, real time RT–PCR and ELISA analyses were used to detect *bcl-2* mRNA and Bcl-2 protein, respectively, in 11 different breast cancer cell lines after hPRL or hPRL-G129R treatment. Our data suggests that Bcl-2 is up-regulated in response to hPRL stimulation and is competitively inhibited by hPRL-G129R in the majority of the cell lines tested. Thus, we propose that the anti-apoptotic role of hPRL in breast cancer is mediated, at least in part, through regulation of Bcl-2.
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Keywords: prolactin; prolactin antagonist; Bcl-2; breast cancer; apoptosis

Introduction

Human PRL is a neuroendocrine polypeptide hormone primarily produced by the lactotrophs in the anterior pituitary gland of vertebrates. It is well established that hPRL is directly involved in the development and differentiation of normal mammary gland in mammalian species (Blackwell and Hammond, 1999; Clevenger *et al.*, 1995; Nagasawa *et al.*, 1985; Topper and Freeman, 1980; Vonderhaar, 1998). Controversy, however, still exists regarding the role of hPRL in human breast cancer. Emerging evidence links hPRL to human breast cancer including: (a) the detection of biologically active hPRL in human breast cancer cells, which suggests that hPRL is produced locally as an autocrine/paracrine growth factor within the mammary gland (Clevenger *et al.*, 1995; Ginsburg and Vonderhaar, 1995; Goffin *et al.*, 1996, Goffin and Kelly, 1997; Vonderhaar, 1999); (b) PRL receptor (PRLR) levels are significantly higher in human breast cancer cells than in normal breast epithelial cells (Kelly *et al.*, 1991); (c) transgenic mice over expressing hPRL have a higher breast cancer incidence (Wennbo *et al.*, 1997) and (d) the hPRL antagonist, hPRL-G129R, slows the growth rate of human breast cancer xymographs in nude mice (Chen *et al.*, 2002). These examples support hPRL's role as a mitogen in human breast cancer and suggest that its antagonist may have potential in treating human breast cancer.

Apoptosis plays a critical role in the regulation of cells that are either in a normal or cancerous state of growth. Key regulators that control apoptosis are kept highly controlled by the cells' internal machinery. One of the first and most widely studied regulators of apoptosis to be identified was Bcl-2, which is now known to be a part of a family of related proteins (Adams and Cory, 1998). *bcl-2* is a human proto-oncogene that when overexpressed, will ultimately lead to the inhibition of cell death (Korsmeyer, 1999). It suppresses apoptosis by blocking the release of cytochrome *c*, a major component of cellular respiration, from the mitochondria, thus preventing the activation of caspases, a group of proteases that carry out the process of cell death (Kumar *et al.*, 2000; Yin *et al.*, 1994). In human breast cancer cells, Bcl-2 and Bax, the inhibitor of Bcl-2, are constitutively expressed to tightly regulate apoptosis (Adams and Cory, 1998; Binder *et al.*, 1996; Kumar *et al.*, 2000; Yin *et al.*, 1994). One of many factors leading to breast malignancy is the up-regulation of *bcl-2* gene expression, ultimately resulting in the inhibition of apoptosis (Green and Beere, 1999). There are numerous molecules that can regulate Bcl-2. For example, IL-3 has been shown to increase the expression of *bcl-2* in hematopoietic cell lines (Krumenacker *et al.*, 1998). Studies using Nb2 cells, a rat lymphoma cell line, show that *bcl-2* is up-regulated in immortalized cell lines (Krumenacker *et al.*, 1998; Leff *et al.*, 1996). One of the more relevant studies involving *bcl-2* demonstrated that treatment of Nb2 cells with PRL results in *bcl-2* up-regulation and *bax* down-regulation (Krumenacker *et al.*, 1998). However, there have been no definitive studies linking hPRL to Bcl-2 activity in human breast cancer cells.

The identification of specific genes that are differentially expressed in response to exogenous treatments has been a subject of great interest to many researchers in the past. There are several methods to compare gene

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expression patterns in tissue cells, such as representational difference analysis, differential display, cDNA array hybridization and serial analysis of gene expression (DeRisi *et al*., 1996; Guiliano *et al*., 1999; Hakvoort *et al*., 1994; Oh *et al*., 1999; Zhan *et al*., 1997). All these methods are able to detect different gene expression profiles, but a newly described technique called suppression subtractive hybridization (SSH) offers additional advantages (Kuang *et al*., 1998; Yang *et al*., 2000). Briefly, SSH first uses mRNA from two populations of cells and converts them into cDNA. The cDNA from cells that contain differentially expressed genes is referred to as the 'tester' and the reference cDNA is referred to as the 'driver'. Both 'tester' and 'driver' cDNAs are first digested using a 4 base-cutter restriction enzyme to create shorter blunt-ended molecules. The ends of the tester cDNAs are modified by ligating adaptors that will serve as PCR primers. The 'tester' cDNAs are then hybridized with 'driver' cDNAs, which have no adaptors on their ends. Suppression PCR, using the adaptors as primers, is then performed to allow exponential amplification of the differentially expressed genes. SSH allows investigators to identify which genes are being turned on or off in one cell type versus another more quickly and easily than other techniques. It is also possible to compare expression profiles of the same cell line by treating a group of cells with a specific compound and using an untreated group as the control. This variation of SSH allows investigators to understand which genes are being expressed in response to a specific treatment of choice. SSH is valuable because it includes an amplification step and selection step that other methods do not, thus increasing the levels of differentially expressed genes while decreasing the levels of housekeeping genes that result in unnecessary background. The introduction of the cDNA microarray makes it possible to identify genes in a much more efficient manner. This emerging technique has proven to be an essential tool when attempting to identify which genes are responding to a certain condition (Oh *et al*., 1999; Yang *et al*., 2000). By combining these two methods it is possible to obtain and identify differentially expressed genes with precision (Beck *et al*., 2001).

In this study, we examined the profile of apoptosis related genes expressed by four human breast cancer cell lines upon treatment with either hPRL or hPRL-G129R. It was found that *bcl-2* gene expression was increased following treatment of breast cancer cells with hPRL in both estrogen receptor (ER) positive cell lines and one of two ER negative cell lines tested. To confirm the evidence linking hPRL and Bcl-2, a quantitative method of RT–PCR and a Bcl-2 ELISA were used to measure both *bcl-2* mRNA expression levels and protein levels in 11 human breast cancer cell lines after treatment with hPRL or hPRL-G129R. The data from these studies suggests that hPRL acts as an apoptosis inhibitor by increasing the expression of Bcl-2 in human breast cancer and that hPRL-G129R competitively inhibits Bcl-2 induction by hPRL.

Results

Profile of apoptosis genes in response to hPRL or hPRL-G129R in four human breast cancer cell lines

Comparisons of the relevant apoptosis related genes expressed in human breast cancer cells are shown in Figure 1. hPRL-G129R treated T-47D and MCF-7 cells shown in Figure 1a,c and hPRL treated in Figure 1b,d, respectively. It appears that in both cases the only gene that was up regulated is *bcl-2* (10-F). In Figure 1a, T-47D cells treated with hPRL-G129R exhibited a strong up-regulation of caspases, such as caspases-3 (11-A), -4 (11-F), -7 (11-I), -9 (11-L) and -10 (11-M). The Bcl-2 binding protein, $BNIP_3$, was expressed in all but the MDA-MB-468 after hPRL-G129R treatment (Table 1). In the T-47D cells, the genes related to death receptors such as serine-threonine kinase 1 (12-H), DAXX (12-I), tumor necrosis factor-related apoptosis inducing ligand (14-F) and death domain receptor 3 (15-G) were up-regulated after hPRL-G129R treatment, although the gene for caspase-8 (11-J and 11-K), which is normally associated with death receptors, has not. In MCF-7 cells the gene *BAD* (10-O in Figure 1c), an important member of the Bcl-2 family of proteins, was differentially expressed after hPRL-G129R treatment. There was no evidence of caspase expression in MCF-7 cells after hPRL-G129R treatment.

Table 1 summarizes all apoptosis related differentially expressed genes in T-47D, MCF-7, BT-549 and MDA-MB-468 cells treated with either hPRL or hPRL-G129R that were probed on an apoptosis microarray. To our knowledge, this is the first time that a list of apoptosis related differentially expressed genes has been compiled for these four breast cancer cells after treatment with hPRL and its antagonist.

Quantitative RT–PCR measurement of bcl-2 mRNA in 11 human breast cancer cell lines

To confirm that hPRL induced the expression of *bcl-2*, quantitative real time RT–PCR was used. Figure 2a represents direct real time RT–PCR output from T-47D cells treated with either hPRL or hPRL-G129R and compared to the untreated control. *bcl-2* message levels were clearly elevated in the hPRL treated samples as indicated by the amplification curves shift to the left and decrease in the hPRL-G129R treated samples as indicated by the amplification curves shift to the right, relative to untreated samples. All samples were normalized to equivalent levels of β-actin mRNA (Figure 2b). Table 2 represents the *bcl-2* levels from multiple quantitative real time RT–PCR runs relative to normalized levels of β-actin. The data is presented as levels of *bcl-2* in all 11 cell lines treated with hPRL, hPRL-G129R or a combination of hPRL and hPRL-G129R, and were compared to levels of *bcl-2* in the untreated controls. The responses are graphed as the per cent change of the experimental response to the untreated control $\pm$s.e. and shown in Figure 3. In MCF-7 and MDA-MB-134 cells, hPRL treatment

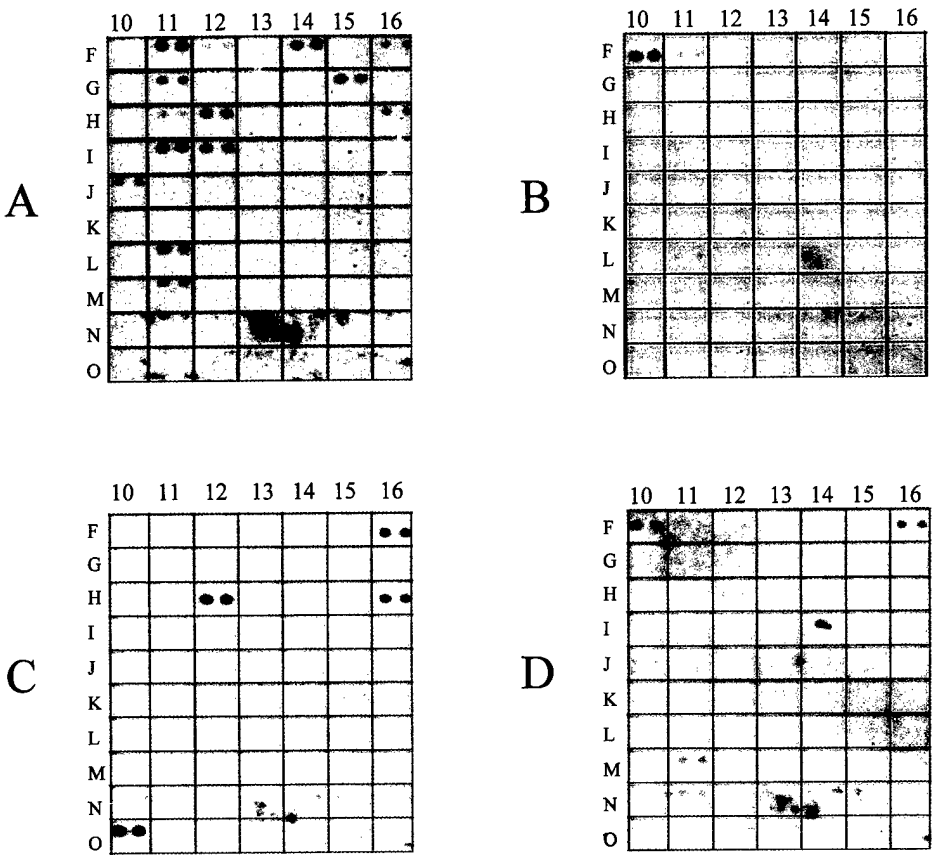

Figure 1 Representation of SSH and microarrays. Three micrograms of DIG labeled differentially expressed cDNAs were probed onto each microarray and detected by means of chemiluminescence. T-47D cells treated with hPRL-G129R (**a**) and hPRL (**b**) and MCF-7 cells treated with hPRL-G129R (**c**) and hPRL (**d**) are shown as a representative of the microarrays

resulted in a highly significant ($P<0.01$) up-regulation of *bcl-2* message, while in BT-549 and T-47D cells the per cent change was significant ($P<0.05$). In the remaining seven cell lines, *bcl-2* message levels were not significantly different from the untreated controls. Treatment with the antagonist resulted in significantly ($P<0.05$) decreased expression of *bcl-2* message in four of the cell lines (MCF-7, T-47D, BT-549 and MDA-MB-157) with no significant change in the other cell lines. A modest increase in *bcl-2* message expression was observed in four cell lines (MDA-MB-436, MDA-MB-468, MDA-MB-231, BT-483) following hPRL-G129R treatment. In seven cell lines a combination treatment of hPRL-G129R and hPRL resulted in lower levels of *bcl-2* expression than the hPRL treatment alone (MCF-7, T-47D, MDA-MB-134, MDA-MB-453, BT-474, MDA-MB-231 and BT-483). The combination treatment significantly ($P<0.05$) reduced *bcl-2* expression levels in MCF-7 and T-47D cell lines, whereas MDA-MB-134, MDA-MB-453, BT-474, MDA-MB-231 and BT-483 cells show an insignificant decrease in the level of bcl-2.

Overexpression of Bcl-2 protein in various cell lines

To further confirm that Bcl-2 was upregulated in human breast cancer cells, a Bcl-2 ELISA was performed on all 11 cell lines. Data is presented as per cent change of Bcl-2 levels in cells treated with hPRL (100 ng/ml) or in cells treated with hPRL-G129R (500 ng/ml) and the combination of hPRL (100 ng/ml) and hPRL-G129R (500 ng/ml) over cells with no treatment. Figure 4a illustrates that six of the 11 human breast cancer cell lines tested showed a highly significant increase ($P<0.01$) in Bcl-2 protein levels after treatment with hPRL. T-47D, MDA-MB-157 and MDA-MB-134 demonstrated the highest levels of Bcl-2 with an increase of approximately 175% over untreated cells. MCF-7, BT-549 and MDA-MB-483 all exhibited levels of Bcl-2 with an increase of approximately 125% over the untreated cells. MDA-MB-468, MDA-MB-453 and BT-474 demonstrated a less significant ($P<0.05$) per cent change of Bcl-2 levels after treatment with hPRL. The remaining two cell lines, MDA-MB-436 and MDA-MB-231, did not demonstrate a significant level of Bcl-2 increase after treatment with hPRL. It is clear from Figure 4b that upon treatment with hPRL-G129R (500 ng/ml), there was a highly significant ($P<0.01$) decrease in the levels of Bcl-2 in all 11 cell lines. The combination treatment of hPRL and hPRL-G129R demonstrated a highly significant ($P<0.01$) decrease of Bcl-2 protein in nine of the 11 cell lines (Figure 4c). MDA-MB-436 and BT-

Table 1 Differentially expressed gene profile for four human breast cancer cell lines

	Cell lines and their treatments							
	(PRLR+ and ER+)				*(PRLR− and ER−)*			
	T-47D		*MCF-7*		*BT549*		*MDA-MB-468*	
Genes	*hPRL*	*G129R*	*hPRL*	*G129R*	*hPRL*	*G129R*	*hPRL*	*G129R*
Cell cycle-regulating proteins and kinases								
Cell division cycle (CDC)-like kinase 1						−		
Serine threonine protein kinase 1	+		+					
Cyclin dependent kinase (CDK)-G2	+						−	
CDK 4 inhibitor 2D						−		
CDC10 protein homolog	+			+	+		−	
Ubiquitin-conjugating enzyme E2						−		
CDC16HS		+						
MAP kinase 3			+				−	
MAP kinase 1					+			
MAPKK 1		+				−		
MAPKK 5							−	
MAPKK 10							−	
Peptidyl-prolyl cis-transisomerase nima-interacting 1					+			
Retinoblastoma-binding protein 4					+		−	
E2F dimerization partner 1								
Bcl-2 family proteins and caspases		+						
B-cell lymphoma protein 2 (Bcl-2)	+		+		+			
Bcl-2-associated death promoter (BAD)				+		+		−
Bcl-2 binding protein ($BNIP_3$)		+		+		−		
BAK1								−
BID3		+						
Caspase-3		+				−		
Caspase-4		+						
Caspase-7		+				+		−
Caspase-8								−
Caspase-9		+						
Caspase-10		+						
Death receptors ligands and apoptosis associated proteins								
TNF receptor 1 associated death domain protein				+		−		
TNF receptor-associated factor 6						+		
Death receptors ligands and apoptosis associated proteins								
Receptor interacting protein (RIP)		+		+		−		−
DAXX		+						−
TNF-alpha converting enzyme		+						
TNF-related apoptosis inducing ligand		+						
Caspase Death Domain								−
Death domain receptor 3		+						
Insulin-like growth factor-binding protein 2 (IGFBP-2)	+			+				
IGFBP-4				+				
Fas-activated serine threonine kinase (FAST)		+		+				
Nuclear kappa factor-B DNA binding subunit			+					
Glutathione peroxidase 1						−		
Glutathione S-transferase theta 1						−		

Cells were either treated with hPRL or hPRL-G129R as indicated. Genes that were differentially expressed are represented with a '+' symbol below the treatment that stimulated their expression and when not expressed the field was left blank as shown

474 cells show a less significant ($P<0.05$) per cent change of Bcl-2 levels after the combination treatment.

Discussion

Apoptosis, or programmed cell death, is a means of regulating cellular growth and differentiation without the inflammatory response generally induced by necrotic cell death (Adams and Cory, 1998). During mammary gland development, and more importantly involution, key apoptosis-inducing Bcl-2 family proteins, such as Bax, Bad and Bcl-w are up regulated (Li, 1997; Schorr *et al*., 1999a), and Bcl-2 appears to act as a regulator of Bax levels. It has been well established that decreased levels of Bax are correlated to increased levels of Bcl-2 and that this Bax Bcl-2 ratio is also critical to normal breast development (Reed, 1998; Green, 2000; Adams and Cory, 1998). Increases in levels of Bcl-2 appear to be more important to cell survival than the down-regulation of Bax (Schorr *et al*., 1999b). The *bcl-2* oncogene has been shown to have an anti-apoptotic function and may play a role in tumorigenesis by raising the threshold for apoptosis (Adams and Cory, 1998). In our previous studies, we reported that an hPRL

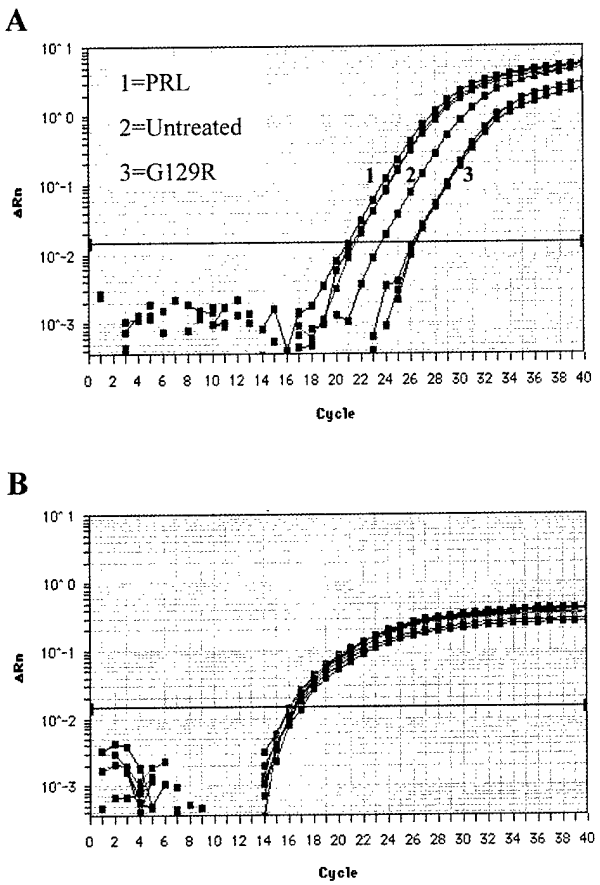

Figure 2 Real time RT–PCR spectral output measuring *bcl-2* levels in T-47D cells treated with 500 ng/ml hPRL (left-hand curve) or 500 ng/ml of hPRL-G129R (right-hand curve), relative to the untreated control (central curve) (**a**). (**b**) Represents the Real time RT–PCR spectral output measuring β-actin levels in untreated, hPRL and hPRL-G129R treated T-47D cells

Table 2 Fold difference of *bcl-2* message of treatments over untreated cells

Cell line	*PRL*[a]	*G129R*[b]	*PRL+G129R*[c]
MCF-7	2.25±0.19	0.50±0.08	0.45±0.15
MDA-MB-134	1.78±0.27	0.82±0.12	0.76±0.17
T47-D	1.59±0.29	0.43±0.15	0.24±0.03
BT549	1.46±0.14	0.30±0.07	1.41±0.38
MDA-MB-436	1.42±0.28	1.38	2.77
MDA-MB-468	1.27±0.15	1.26±0.09	1.44±0.09
MDA-MB-157	1.22±0.05	0.33±0.07	1.26
MDA-MB-453	1.04±0.16	0.78±0.22	0.75
BT474	0.87±0.11	0.82±0.15	0.88±0.27
MDA-MB-231	0.79±0.06	1.21	0.43
BT483	0.75	1.41	0.55

Numbers represent real time RT–PCR data correlating to Figure 3 and represent *bcl-2* message levels. Cells were either treated with hPRL (500 ng/ml) or hPRL-G129R (500 ng/ml). Combination treatement (*PRL+G129R*) is as follows: 200 ng/ml PRL+1000 ng/ml G129R for 48 h. All values are represented as fold change over the untreated controls and are mean±s.e. [a]$n=2-5$; [b]$n=2-4$; [c]$n=2-3$

antagonist, hPRL-G129R, is able to inhibit human breast cancer cell proliferation through the induction

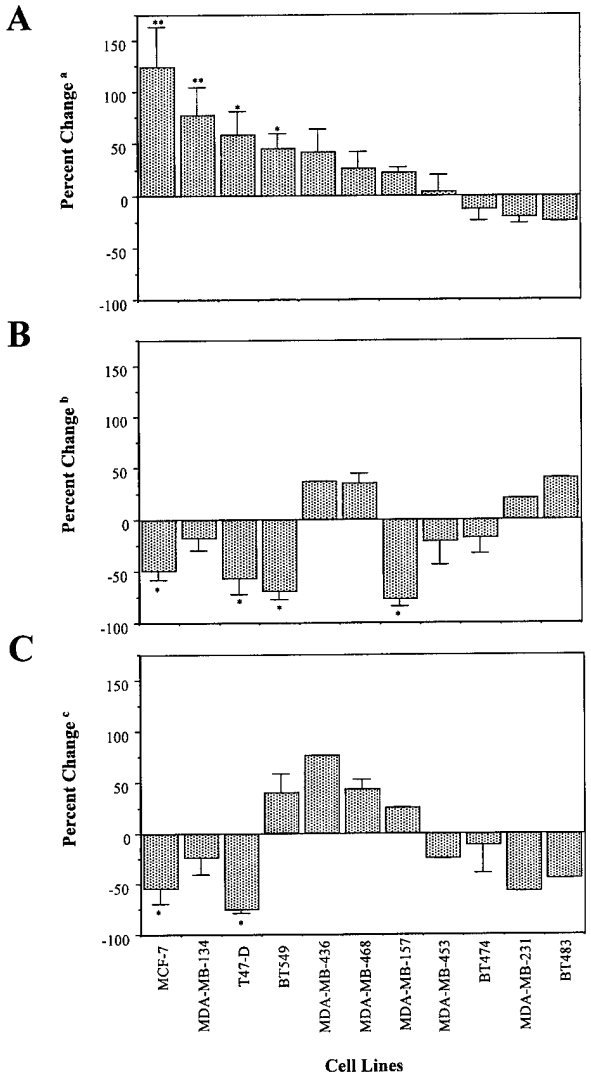

Figure 3 Real-time quantitative measurement of *bcl-2* mRNA levels in 11 breast cancer cell lines in response to 48 h treatments with hPRL (500 ng/ml; **a**), hPRL-G129R (500 ng/ml; **b**) and a combination treatment of hPRL (200 ng/ml) and hPRL-G129R (1000 ng/ml; **c**). Levels are represented as the fold change over the untreated controls, and numbers are presented as mean±s.e. **a**, $n=2-5$; **b**, $n=2-4$; **c**, $n=2-3$. $^{**}P<0.01$ *versus* basal levels of Bcl-2; $^{*}P<0.05$ *versus* basal levels of Bcl-2

of apoptosis (Chen *et al*., 1999), suggesting that the role of hPRL in breast cancer cells may be anti-apoptotic. We have also shown that hPRL down-regulates TGFβ1 (apoptotic factor) and up-regulates TGFα (survival factor) secretion in a dose-dependent manner in human breast cancer cells (Ramamoorthy *et al*., 2001). More importantly, hPRL-G129R up-regulates TGFβ1 and down-regulates TGFα. In the same study it was also shown that caspase-3 is up regulated by hPRL-G129R. In the present study, we looked at a vast array of genes within breast cancer cells that are responding to treatment with hPRL and hPRL-G129R. We provide evidence that the potential tumorigenic effects (autocrine and paracrine) of hPRL may be mediated through the up-regulation of Bcl-2.

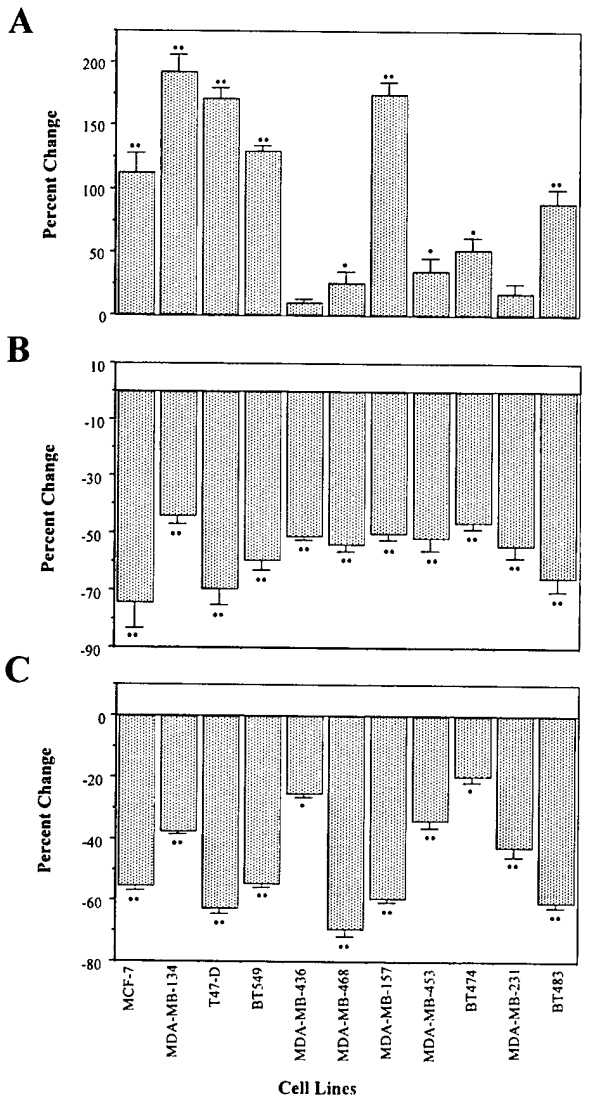

Figure 4 Expression of the Bcl-2 protein in various cell lines. Cells were treated with either hPRL (100 ng/ml; **a**), hPRL-G129R (500 ng/ml; **b**) or a combination of hPRL (100 ng/ml) and hPRL-G129R (500 ng/ml, **c**) for 48 h, lysed and ELISAs were performed to determine the relative levels of Bcl-2 present in the cells. All cells were assayed in triplicate and levels of Bcl-2 was determined using a standard curve prepared using known Bcl-2 standards from the manufacture. Fold induction and inhibition were determined using untreated cells as a control for hPRL treated cells and hPRL treated cells as a control for the combination treatment. Cell lines are arranged from left to right in order of increasing bcl-2 mRNA message level (Figure 3.). Numbers are presented mean ± s.e. of at least three independent experiments. $^{**}P<0.01$ *versus* basal levels of Bcl-2; $^{*}P<0.05$ *versus* basal levels of Bcl-2

The microarray studies using four breast cancer cell lines implied the heterogeneous nature of human breast cancer (Table 1). It is apparent in the gene expression patterns of four cell lines that the gene *bcl-2* is overexpressed in T-47D, MCF-7 and BT-549 breast cancer cells following hPRL treatment. In response to hPRL-G129R treatment, BNIP3, a 19-KD dimeric mitochondrial protein that binds to Bcl-2 and suppresses its anti-apoptotic activity (Chen *et al*., 1997), is up-regulated in three of the four cell lines (Table 1). These data suggest an additional role for hPRL in regulation of Bcl-2 activity in breast cancer cells. To further confirm this finding, we utilized 11 breast cancer cell lines and treated them with hPRL, hPRL-G129R or a combination of the two and measured *bcl-2* mRNA as well as Bcl-2 protein levels. Increases in *bcl-2* mRNA levels in four cell lines (MCF-7, MDA-MB-134, T-47D and BT-549) are highly significant ($P<0.01$) following treatment by hPRL and are down regulated by hPRL-G129R treatment in five cell lines (MCF-7, MDA-MB-134, T-47D and BT-549 and MDA-MB-157) (Figure 3). However, the response of Bcl-2 to the treatment of hPRL or hPRL-G129R was most apparent at the Bcl-2 protein level (Figure 4). Eight of the 11 cell lines demonstrated a highly significant increase of Bcl-2 protein after hPRL treatment ($P<0.01$). The maximum increase in Bcl-2 protein after a single dose treatment with hPRL was several fold higher than the basal level in untreated controls. In contrast, the decrease of Bcl-2 levels in response to treatment by hPRL-G129R was highly significant ($P<0.01$) in all 11 cell lines (Figure 4). The cell lines (MDA-MB-436, MDA-MB-468, MDA-MB-453, BT-474, MDA-MB-231 and BT-483) demonstrated no statistically significant *bcl-2* response to hPRL treatment at the mRNA level, and showed a relatively lower response at the protein level. This would suggest that in these cell lines hPRL plays a less significant role in maintaining proliferation via the Bcl-2 protein. The discrepancy between the Bcl-2 mRNA levels and the protein levels in some cell lines after treatment may be attributed to many factors, which include the general instability of mRNA in the cells, relative to protein, where Bcl-2's half-life is greater than 10 h (Merino *et al*., 1994).

The cell lines used in the cDNA subtraction experiments have been reported to produce PRL as an autocrine/paracrine growth factor, from the work of others (Ginsburg and Vonderhaar, 1995; Shaw-Bruha *et al*., 1997; Yamauchi *et al*., 2000). We do not have evidence, at this point, that hPRL-G129R contains an intrinsic ability to elicit novel signal transduction of its own. Rather, we believe that the down regulation of the *bcl-2* expression by hPRL-G129R is through the competitive inhibition of the effects induced by endogenous PRL. These findings are consistent with those of Llovera *et al*. (2000), who have found that the hPRL-G129R does not activate any specific signaling molecules in their systems.

We found no obvious correlation between the published (Ormandy *et al*., 1997) ER status and the *bcl-2* response following hPRL and hPRL-G129R treatment. For example, both the ER negative BT-549 and the most ER positive MDA-MB-134 cell lines demonstrated high levels of *bcl-2* response, while the strongly ER positive cell line BT-483 and the ER negative MDA-MB-231 cell lines had the lowest responses. In our previous work, we determined relative PRLR mRNA expression levels in 11 breast

cancer cell lines (Peirce and Chen, 2001). In this study, the levels of *bcl-2* expression do not appear to have a linear correlation with that of PRLR mRNA. This might be attributed to the fact that there are multiple intracellular signaling mechanisms involved in regulation of *bcl-2* expression. For example, there is recent evidence of cross-talk between PRL receptor and HER2/neu through phosphorylation of Jak2 that leads to the activation of MAP kinases in breast cancer cell lines (Yamauchi *et al*., 2000). Related to this finding, we have seen that MAP kinases are, in fact, up regulated by PRL treatment in three out of four cell lines (Table 1). Further work in this area should help to identify the specific pathways by which cellular apoptosis is regulated by hPRL and hPRL-G129R.

cDNA microarrays provide a powerful means for identifying genes differentially expressed in cells after certain alterations. However, the vast amount of information revealed after array analyses often leaves more questions than answers. In this study, we decided to focus on one gene, *bcl-2*, after analysing initial results of the cDNA subtraction and array analysis. There are clearly many questions to be addressed (Table 1). For example, we found that there was no evidence of caspase expression in MCF-7 cells following treatment by hPRL-G129R, whereas caspase expression is up regulated in T-47D, BT-549 and MDA-MB-468 cells. We previously reported that caspase-3 activity was up regulated in T-47D cells following treatment with hPRL-G129R (Ramamoorthy *et al*., 2001). There is a lack of caspase mRNA expression and the appearance of a more direct link to Bcl-2 related apoptosis via the BAD (Bcl-2 associated death promoter) protein in MCF-7 cells (Figure 1c). During apoptosis, BAD has been shown to bind to Bcl-2 and release it from the mitochondrial membrane resulting in total cellular disruption. BAD operates upstream of the caspase pathway suggesting that MCF-7 cells activate apoptosis via a different pathway utilized by the other three breast cancer cell lines. It has been previously shown that MCF-7 cells lack caspase-3 entirely due to a 47-base pair deletion within the CASP-3 gene, although this cell line is still able to undergo apoptosis even in the absence of DNA fragmentation (Janicke *et al*., 1998; Liang *et al*., 2001). Thus, our data tentatively identifies one component of the caspase-3 independent signaling pathway that MCF-7 cells may use to trigger apoptosis. It is also interesting to point out that a death domain protein, the receptor-interacting protein (RIP), is differentially expressed in all four cell lines following hPRL-G129R treatment. It has been reported that over-expression of RIP induces both NF-κB activation and apoptosis (Hsu *et al*., 1996). Our results may be of interest when investigating the death domain proteins and death domain receptors in relation to hPRL and hPRL-G129R.

In summary, a list of apoptosis related genes that are differentially expressed following treatment with either hPRL or hPRL-G129R has been compiled for four different breast cancer cell lines. These data will allow for future studies of specific genes that are involved with cellular proliferation or apoptosis in human breast cancer. By focusing on Bcl-2 mRNA or protein expression in response to hPRL and hPRL-G129R treatment in 11 cell lines, we provide further evidence that the anti-apoptotic effects of hPRL in breast cancer are likely mediated through the up regulation of Bcl-2. It is generally accepted that, for cancer therapy, one should not design an approach based solely upon increasing death signals, such as chemotherapeutics. Rather, a twofold approach combining chemotherapeutics with removal of survival factors will result in a more efficient treatment. Our data regarding hPRL-G129R further strengthens its potential therapeutic role in breast cancer therapy.

Materials and methods

Cell lines and growth conditions

Five ER positive (T-47D, MDA-MB-134, BT-474, MDA-MB-483, MCF-7) and six ER negative human breast cancer cell lines (BT-549, MDA-MB-231, MDA-MB-453, MDA-MB-468, MDA-MB-436 and MDA-MB-157) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). T-47D, BT-549, BT-474 and MDA-MB-483 cells were maintained in RPMI 1640 medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT, USA) and 100 μg/ml gentamycin (for all media used) (Hyclone). BT-459 cells were supplemented with 200 IU of Insulin (Sigma, St. Louis, MO, USA). MDA-MB-483 cells were supplemented with 0.2 mM sodium pyruvate, 2 mM HEPES buffer and 200 IU Insulin (Sigma). MCF-7 cells were maintained in DMEM (Life Technologies) supplemented with 10% FBS. MDA-MB-231, MDA-MB-134, MDA-MB-453, MDA-MB-468, MDA-MB-436 and MDA-MB-157 cells were maintained in Leibovitz L-15 (Life Technologies) media. MDA-MB-231, MDA-MB-436 and MDA-MB-453 cells were supplemented with 10% FBS with the addition of 200 IU of Insulin for MDA-MB-436. MDA-MB-468 and MDA-MB-157 cells were grown in the presence of 15% FBS and MDA-MB-134 in the presence of 20% FBS. Cell lines T-47D, BT-549, BT-474, MDA-MB-483, MCF-7 and MDA-MB-231 were grown at 37°C in a humid atmosphere in the presence of 5% CO_2. MDA-MB-134, MDA-MB-453, MDA-MB-468, MDA-MB-436 and MDA-MB-157 cells were grown at 37°C in a humid atmosphere in the absence of CO_2.

PCR-Select cDNA suppression subtraction hybridization

Before experiments, cells were split into three groups of 10 T75 flasks and grown in their specific medium supplemented with 10% charcoal-stripped fetal bovine serum (CSS) until 80% confluent. Approximately 1×10^8 cells from each group were treated with either 500 ng/ml of hPRL (hPRL was kindly supplied by Dr AF Parlow, National Hormone and Pituitary Program, NIH, USA) or 500 ng/ml of hPRL-G129R in cell specific media supplemented with 1% CSS. The untreated control cells were cultured in their respected medium supplemented with 1% CSS. All cells were treated for 48 h and immediately harvested for mRNA extraction. Polyadenylated mRNA was isolated using the Micro-Fast Track 2.0 kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. RNA yield was determined by

measuring absorbency at 260 nm. SSH was performed using the PCR-Select™ cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA) as previously described (Beck *et al*., 2001). Three micrograms of purified cDNAs from the subtraction hybridizations were random primed labeled with alkali labile digoxigenin-dUTP using the DIG DNA Labeling Kit (Roche Molecular Biochemical's, Mannheim, Germany) according to the manufacturer's protocol.

cDNA microarrays

The Atlas™ human apoptosis array (Clontech) was used for all microarray analyses. The array is a nylon membrane that contains all known apoptosis related genes (205 cDNAs) spotted onto the surface in duplicate. Membranes were prehybridized with DIG Easy Hyb solution (Roche) overnight at 37°C in a hybridization incubator with gentle rotation. DIG-labeled probes were purified and resuspended appropriately in TE (pH 8.0). The probes were boiled for 10 min and placed on ice for 5 min. After prehybridization, the DIG-labeled probe was added to the microarray membrane in a total volume of 5 ml of fresh DIG Easy Hub and allowed to hybridize overnight at 68°C in a hybridization incubator with gentle rotation. Membranes were washed twice at 38°C for 5 min in 2× SSC, 1% SDS, and twice at 68°C for 15 min in 0.1× SSC, 0.5% SDS. DIG-labeled cDNAs on the hybridized Atlas™ membranes were detected by chemiluminescence using the DIG luminescent detection kit (Roche) according to the manufacturer's specifications using CSPD® as the chemiluminescent substrate. After incubation with CSPD®, membranes were placed in an autoradiography cassette and incubated at 37°C for 15 min to enhance the exposure and then exposed to Kodak Biomax™-MR film at room temperature (all hybridizations were carried out in duplicate). It was determined that using 3 μg of differentially expressed cDNAs for labeling and probing the microarrays was the correct amount for less background and optimal brightness for gene identification. Each array was exposed to the film for various periods of time to allow for the correct exposure to be captured.

Real time quantitative RT–PCR

Before treatment, 11 breast cancer cell lines were depleted of serum for 3 to 4 days in their respective medium supplemented with 1% CSS. Approximately $0.5-1\times7^7$ cells from each group were treated with 500 ng/ml of hPRL, 500 ng/ml of hPRL-G129R or a 1000 ng : 250 ng ratio of hPRL-G129R to hPRL in cell specific medium supplemented with 1% CSS. The untreated control cells were cultured in cell specific medium supplemented with 1% CSS. All cells were treated for 48 h and harvested for total RNA extraction using the RNAqueous (Ambion, Austin, TX, USA) RNA isolation kit.

A one-step real time reverse transcription PCR (RT–PCR) technique was used to determine relative expression levels of *bcl-2* mRNA using the ABI Perkin Elmer Prism 7700 Sequence Detection System (Perkin-Elmer Biosystems, Foster City, CA, USA). The reaction mix included a pre-developed TaqMan® assay mixture containing both forward and reverse *bcl-2* specific primers and a 100 nm final concentration of the *bcl-2*-specific probe labeled with FAM reporter fluorescent dye (Perkin-Elmer 4319432F). A one-step reaction mixture provided in the TaqMan® Gold RT–PCR Kit (Perkin-Elmer) was used for all amplifications (5.5 mM $MgCl_2$, 50 mM KCl, 0.01 mM EDTA, 10 mM Tris-HCl pH 8.3, 300 μM deoxyATP, 300 μM deoxyCTP, 300 μM deoxyGTP, 600 μM deoxyUTP, 0.025 U ml AmpliTaq Gold DNA polymerase, 0.25 U/ml MultiScribe Reverse Transcriptase, 0.4 U/ml RNase inhibitor). Cycle parameters for the one-step RT–PCR included a reverse transcription step at 48°C for 30 min followed by 40 cycles of 95°C denaturation and 60°C annealing/extension. Four hundred to 1500 ng of total RNA was used per reaction. The housekeeping gene β-actin was used for internal normalization. Each reaction was carried out in triplicate for each PCR run, and each run was repeated two to five times. Data are expressed as the mean ± s.e.

Bcl-2 ELISA

A Bcl-2 ELISA (Oncogene Research Products, Boston, MA, USA) analysis was performed according to manufacturer's instructions. Briefly, all 11 cell lines were treated with hPRL (100 ng/ml), hPRL-G129R (500 ng/ml) or combination of hPRL (100 ng/ml) and hPRL-G129R (500 ng/ml) for 48 h in depleted media specific for each cell line described previously. Controls for all treatments were those of untreated cells in their specific defined media. For each cell line, approximately 5×10^6 cells were resuspended in 1 ml of resuspension solution (50 mM Tris, 5 mM EDTA, 0.2 mM PMSF, 1 μg/ml pepstatin and 0.5 μg/ml leupeptin; pH adjusted to 7.4). Two hundred microliters of antigen extraction agent (Oncogene) were added to the cell suspensions and incubated on ice for 30 min. Cell debris was centrifuged and supernatant was frozen until use. Each supernatant was diluted 1 : 1 to obtain an optimal reading in the range of the standards. Standards were performed in duplicate. Analyses were repeated three times and data are expressed as the mean ± s.e.

Statistical analyses

All values are given as mean ± s.e. Statistical analysis was performed using the program StatsDirect version 1.9.8 (CamCode, Cambridge, UK) with one way ANOVA and a Tukey-Multiple Comparison test. *P*-values less than 0.01 were considered highly statistically significant and *P* values that were less than 0.05 were considered statistically significant.

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Prolactin Antagonist-endostatin Fusion Protein as a Targeted Dual-Functional Therapeutic Agent for Breast Cancer[1]

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ABSTRACT

In previous studies (Chen, W. Y. *et al.*, Clin. Cancer Res., *5:* 3583–3593, 1999; Chen, N Y. *et al.*, Int. J. Oncol., *20:* 813–818, 2002), we have demonstrated the ability of the human prolactin (hPRL) antagonist, G129R, to inhibit human breast cancer cell proliferation *in vitro* and to slow the growth rate of tumors in mice. We further revealed that the possible mechanisms of G129R antitumor effects act through the induction of apoptosis via the regulation of *bcl-2* gene expression. It has been established that to sustain tumor growth, it is necessary for the development of a network of blood vessels to bring in nutrients, a process called angiogenesis. The disruption of angiogenesis has been proven to be an effective strategy to cause regression of certain tumors. One of the best-studied angiogenesis inhibitors is endostatin, which acts through the inhibition of endothelial cells. In this study, we combine the anti-breast tumor effects of G129R and the antiangiogenic effects of endostatin by creating a novel fusion protein (G129R-endostatin) specifically for breast cancer therapy. The data presented here demonstrated that this novel fusion protein was able to bind to the PRL receptor (PRLR) on T-47D human breast cancer cells and inhibit the signal transduction induced by PRL. At the same time, G129R-endostatin inhibited human umbilical vein endothelial cell (HUVEC) proliferation and disrupted the formation of endothelial tube structures with potency similar to that of endostatin. More importantly, the therapeutic efficacy of G129R-endostatin was confirmed using a mouse breast cancer cell line 4T1 *in vivo*. G129R-endostatin has a significantly prolonged serum half-life as compared with that of G129R or endostatin alone, and exhibited greater tumor inhibitory effects than G129R and endostatin individually or in combination. Taken together, these data demonstrate the dual therapeutic effects of G129R-endostatin, and suggests that this fusion protein has great promise as a novel anti-breast cancer agent.

INTRODUCTION

Human breast cancer affects ~1.1 million women per year, and ~35% of these new cases will eventually result in death. Tumor metastasis still remains the main cause of breast cancer deaths (1). Although with chemotherapy and radiation therapy, the prognosis has improved in some cases, these approaches may result in severe side effects. Recently, PRL[3] has become one of the focal points in the investigation into the mechanism and onset of human breast cancer (2, 3). hPRL has been linked to breast cancer by several lines of evidence: (*a*) an autocrine/paracrine loop for hPRL has been demonstrated with the finding of biologically active PRL in breast cancer cells (2, 4–7); (*b*) PRLR expression levels are up-regulated in breast cancer cells and neoplastic mammary tissues (8); (*c*) there is a high breast cancer rate in transgenic mice overexpressing lactogenic hormones (9); and (*d*) inhibition of PRL activity with an antagonist inhibits the proliferation of breast cancer cells both *in vitro* (10) and in mouse studies (11). In view of these studies, it is evident that PRL plays an important etiological role in breast cancer, and that the development of a PRL receptor antagonist may have potential as a therapeutic agent in treating this disease.

In previous studies, it was demonstrated that a single amino acid substitution mutation in hPRL resulted in a PRLR antagonist, G129R (5, 10). We have further determined that G129R inhibits human breast cancer cells through the induction of apoptosis (10). One of the key mechanisms that controls signal transduction of breast cancer cells is the stimulation of the JAK/STAT/mitogen-activated-protein-kinase (MAPK) pathways by PRL. Our previous work has shown that G129R inhibits human breast cancer proliferation, at least in part, through the inhibition of STATs phosphorylation (12). In addition, hPRL up-regulates the proapoptotic gene *bcl-2*, and G129R competitively down-regulates the *bcl-2* gene expression in human breast cancer cells (13). Furthermore, anti-breast tumor effects of G129R were confirmed by using human breast cancer xenografts in nude mice (11). These studies provide strong evidence for the ability of G129R to inhibit human breast cancer and the potential to become a therapeutic agent for the treatment of human breast cancer.

A key factor in the maintenance of the uncontrollable growth of cancer cells is the formation of new blood vessels in the tumor mass to provide nutrients, namely tumor angiogenesis (14–17). Angiogenesis is also required for tumor metastasis to occur and, thus, the inhibition of tumor angiogenesis holds great promise as a therapeutic approach in stopping primary tumor growth and metastasis (18). In recent years, there have been several inhibitors of angiogenesis identified including thrombospondin (TSP-1), angiostatin, protamine, and endostatin (19, 20). Endostatin is a M_r 20,000 COOH-terminal fragment of collagen XVIII and was first characterized in murine EOMA cells by O'Reilly *et al.* (21) and was later characterized in humans (22). Endostatin is a specific inhibitor of endothelial cell proliferation and is a potent inhibitor of angiogenesis (23–25). Although the mechanism of endostatin activity is not fully understood, the crystal structure of endostatin reveals a heparin sulfate-binding site (26), suggesting that endostatin can inhibit such heparin-binding angiogenic factors as bFGF-2. Murine tumors that are dependent on angiogenesis for growth were successfully regressed to microscopic lesions after systemic therapy with murine endostatin (21). Such inhibition may lead to tumor dormancy as a result of an increased level of apoptosis in endothelial cells (27). Recently, Phase I clinical trials of endostatin have been completed, and it is currently in Phase II studies. In this study, we combined the tumor targeting and inhibitory activities of G129R with the antiangiogenic abilities of endostatin by creating a novel fusion protein (G129R-endostatin), and tested its potential dual therapeutic effects both in cell culture as well as in mouse tumor models.

MATERIALS AND METHODS

Cell Lines and Growth Conditions. The human breast cancer cell line T-47D, mouse breast cancer cell line 4T1, and HUVECs were purchased from the American Type Culture Collection (Manassas, VA). T-47D cells were maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FBS (Hyclone Laboratories, Logan, UT) and 100 μg/ml

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[3] The abbreviations used are: PRL, prolactin; hPRL, human PRL; PRLR, hPRL receptor; bFGF, basic fibroblast growth factor; HUVEC, human umbilical vein endothelial cell; FBS, fetal bovine serum; IRMA, immunoradiometric assay; TBS, Tris-buffered saline; ECM, extracellular matrix.

gentamicin (Hyclone). 4T1 cells were maintained in RPMI 1640 supplemented with 10% FBS, 1% sodium pyruvate (Life Technologies, Inc.), 100 μg/ml gentamicin, 4.5 g/liter glucose (Sigma, St. Louis, MO), and 1 mM HEPES (Life Technologies, Inc.). HUVECs were maintained in Medium-199 supplemented with 10% FBS, 100 μg/ml gentamicin, and an EGM-2 Singelquot (Cambrex, East Rutherford, NJ). Cells were grown at 37°C in a humid atmosphere in the presence of 5% CO_2.

Cloning and Expression of G129R-Endostatin Fusion Protein. A two-step cloning procedure was used to generate a recombinant cDNA encoding G129R fused to human endostatin. Primers corresponding to G129R (5′ primer; restriction site for *Nde*I underlined, 5′-CAT ATG TTG CCC ATC TGT CCC GGC-3′, and 3′ primer, restriction site for *Bam*HI underlined, 5′-GGA TCC GCA GTT GTT GTT GTG GAT-3′) were used to amplify the G129R fragment from a previous clone (10). Primers corresponding to human endostatin (5′ primer; restriction site for *Bam*HI underlined, 5′-GGA TCC CAC AGC CAC CGC GAC TTC CAG-3′, and 3′ primer, restriction site *Xho*I with stop codon underlined, 5′-CTC GAG CTA CTT GGA GGC AGT CAT GAA GC-3′) were used to amplify the gene from a Human Universal QUICK-Clone cDNA library (Clontech, Palo Alto, CA). Another 5′ primer, *Nde*I, 5′-CAT ATG CAC AGC CAC CGC GAC TTC CAG, was used with the *Xho*I 3′ primer for expression of human endostatin alone. All of the cDNA fragments were ligated separately into the TA cloning vector pCR2.1 (Invitrogen, Inc., Carlsbad, CA), were restriction mapped, and were sequenced. The cDNA fragments were restriction digested at the cloned restriction sites, were purified, and were ligated into the protein expression vector pET22b(+) (Novagen, Madison, WI) for the expression of G129R-endostatin and endostatin proteins. The design of the fusion protein is such that the NH_2-terminal portion of endostatin is ligated to the COOH-terminal portion of G129R.

Production and Purification of Endostatin, G129R, and G129R-Endostatin Fusion Protein. G129R was purified as described previously (10). Endostatin and G129R-endostatin were purified according to Huang *et al.* (28). Briefly, BL21 (Novagen) chemically competent cells were transformed with pET22b(+) vector encoding for endostatin, G129R, and G129R-endostatin cDNA. Bacteria were allowed to grow overnight in Luria-Bertani broth (ampicillin, 50 μg/ml) at 37°C. The next day the bacteria were induced with isopropyl-beta-D-thiogalactopyranoside (IPTG) for 5 h to induce protein expression. Bacteria were collected and were resuspended in 100 ml of buffer A [0.1 M Tris-HCl (pH 8.0) and 5 mM EDTA], followed by incubation at room temperature for 15 min, with the addition of lysozyme at a final concentration of 50 μg/ml. The suspension was then sonicated using a 550 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) in the presence of 0.1% sodium deoxycholate, followed by centrifugation at 8000 × *g* for 10 min. The pellet was resuspended in 100 ml of buffer A containing 0.1% sodium deoxycholate. The centrifugation/resuspension procedure was repeated twice. The pellet was dissolved in 30 ml of buffer B [0.05 M Tris (pH 8.0), 1% SDS, and 1 mM DTT] and was centrifuged at 8000 × *g* for 10 min at 4°C. The clear supernatant obtained was then transferred to dialysis tubing with a M_r cutoff of 10,000 and was dialyzed twice in 1500 ml of buffer C [0.05 M Tris-HCl (pH 8.0) and 0.1 mM DTT] at 4°C for 4 h. The recombinant protein was then further dialyzed twice in 1500 ml of buffer D [0.05 M Tris-HCl (pH 8.0)] and twice in 1000 ml of buffer E [0.05 M Tris-HCl (pH 8.0), 0.01 mM oxidized glutathione, and 1 mM reduced glutathione] at 4°C for 4 h/dialysis cycle, respectively. A final dialysis in 0.05 M Tris-HCl (pH 8.0) was performed overnight. Both endostatin and G129R-endostatin were soluble in the dialysis buffer. The G129R protein was purified on a fast-performance liquid chromatography system (FPLC; Amersham Pharmacia, Newark, NJ) after refolding as described previously (12). The endostatin and G129R-endostatin fusion protein preparations contain ~400 EU/mg protein and G129R preparation contains <5 EU/mg protein as tested by the Gel-Clot method (Cape Cod, Inc). The concentration of G129R, endostatin, and G129R-endostatin was determined by the Bio-Rad protein assay method (Bio-Rad, Hercules, CA) and G129R and G129R-endostatin were further verified using a hPRL IRMA kit (DPC, Inc., Los Angeles, CA). The purity of the proteins was determined on a SDS-PAGE gel stained with Coomassie Blue (Fisher Scientific).

Immunoblot Analysis. G129R, endostatin, and G129R-endostatin were separated on a 4–15% SDS-PAGE gel. The proteins were transferred to enhanced chemiluminescence Hybond nitrocellulose (Amersham Pharmacia) at 12 W for 2 h. The nitrocellulose blot was blocked with TBS containing 0.05% Tween 20 and 5% milk (blocking buffer) for 1 h at room temperature. Blots were incubated overnight at 4°C in blocking buffer containing the appropriate antibody [rabbit antihuman endostatin, 1:200 (Oncogene Research Products, San Diego, CA); rabbit anti-hPRL antiserum, 1:1000 (Dr. A. Parlow, National Hormone and Pituitary Program, NIH, Bethesda, MD)]. The blots were washed three times, 5 min each, with TBS containing 0.05% Tween, and were incubated with the secondary antibody goat-antirabbit horseradish peroxidase (1:5000; Bio-Rad) for 2 h at room temperature with gentle agitation. Blots were washed three times, 5 min each, with TBS containing 0.05% Tween and were developed for 1 min using the ECL Western detection reagents (Amersham Pharmacia). Immunoblots were visualized using Kodak MR film (Fisher).

Radioreceptor Binding Assay. T-47D human breast cancer cells expressing the PRL receptor were grown to confluency (~10^5 cells/well) in six-well tissue culture plates. Cells were starved in serum-free RPMI 1640 for 1 h, and then were incubated for 2 h at room temperature in serum-free RPMI medium containing ^{125}I-labeled hPRL (specific activity, 40 μCI/μg; NEN Perkin-Elmer, Boston, MA) with or without various concentrations of PRL, G129R, endostatin, and G129R-endostatin. Cells were washed three times with serum-free RPMI medium and were lysed in 0.5 ml of 0.1 N NaOH/1% SDS. The bound radioactivity was determined by scintillation counting, and the percentage of specific displacement was calculated and compared among these samples.

Immunofluorescence Staining. T-47D cells and HUVECs were maintained as described previously. Cells were passed onto Lab-Tek Chamber Slide System (Fisher) and were grown to ~70% confluency. HUVECs were cultured in low-serum medium (2% FBS), and T-47D cells were serum depleted for 30 min. Cells were treated with 10 μg/ml (435 nM) of G129R, 10 μg/ml (500 nM) of endostatin, or 20 μg/ml (476 nM) of G129R-endostatin for 30 min at 37°C. Cells were treated in their respective serum-free media, and all of the staining was performed in triplicate and repeated at least twice. After treatment, cells were washed with PBS [120 mmol NaCl; 2.7 mmol KCl; and 10 mmol phosphate buffer salts (pH 7.4)], fixed with 4% *para*-formaldehyde (BD Biosciences, Bedford, MA) for 25 min at 4°C, and permeabilized with 0.2% Triton X-100 in 1× PBS. Cells were incubated in blocking buffer for 30 min with 2% BSA (Fisher). Cells were incubated with the primary antibodies rabbit antihuman endostatin (Ab-2), 1:200, and mouse anti-hPRL antiserum, 1:1000, at room temperature for 2 h. After incubation, cells were washed three times with 1% BSA/PBS and subjected to secondary antibody (1:500) incubation for 2 h at room temperature using Alexa Fluor 594 goat antimouse IgG (red fluorescence) and Alexa Fluor 488 goat antirabbit IgG (green fluorescence; Molecular Probes, Inc., Eugene, OR), respectively. Cells were rinsed twice with 1% BSA/PBS and incubated with Anti-Fade equilibrium buffer (10 μl/well; Molecular Probes) for 10 min at room temperature. The chambers were then removed and cover slides were mounted for observation. All of the wells were examined under an Zeiss LSM 510 confocal microscope using 488-nm and 594-nm wavelengths. Digital photographs were taken at ×450.

STAT-5 Phosphorylation Assay. T-47D cells were grown to 80% confluency in six-well plates in RPMI 1640 containing 10% charcoal-stripped FBS. On the day of the experiment, cells were depleted for 30 min in RPMI 1640 containing 0.5% charcoal-stripped FBS. Cells were then treated for 20 min with the appropriate amount of PRL, G129R, endostatin, G129R-endostatin, or a combination treatment as indicated in Fig. 4. Cells were washed with ice-cold PBS and were lysed with 200 μl of lysis buffer [50 mM Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM Na_3VO_4] and were incubated on an orbital shaker for 10 min at room temperature. The lysate was transferred to a sterile 1.5-ml centrifuge tube, gently passed through a 21-gauge needle six times, and then incubated on ice for 20 min. The lysate was centrifuged at 12,000 × *g* for 20 min at 4°C. The supernatant was removed, and 30 μl of the lysate (65–70 μg) was used for Western blotting analysis as described earlier, with the exception that anti-STAT5A + anti-STAT5B [1:4000; Upstate Biotechnology Inc. (UBI), Lake Placid, NY] or anti-phospho-STAT5 (1:5000; UBI) were substituted as the primary antibodies.

Cell Proliferation Assay. HUVEC's and T-47D cells were grown in their respective media free of phenol-red. Fully confluent HUVEC and T-47D cell cultures were trypsinized, and cells were resuspended in medium containing 5% FBS. Cells were then seeded into 96-well culture plates at a density of 5,000 HUVECs/well [in the presence of 2.5 ng/ml bFGF (Sigma) and 1 μg/ml heparin (Sigma)] and 15,000 T-47D cells/well. After an incubation of 24 h, various concentrations of G129R, endostatin, or G129R-endostatin were added to the appropriate well. Cells were further incubated for 72 h at 37°C in a

humidified 5% CO_2 incubator. The viability of the cells was determined using the MTS-PMS (CellTiter 96 Aqueous kit; Promega Corp., Madison, WI) colorimetric assay (following the manufacturer's instructions), and absorbance at 490 nm was determined using a microplate reader (Bio-Rad). Cell survival was calculated as a percentage of the control values. All of the experiments were carried out in triplicate.

Endothelial Tube Formation Assay. Matrigel (BD Biosciences) was added (320 μl) to each well of a 24-well plate and allowed to polymerize at room temperature for 20 min. A suspension of 30,000 HUVECs/well in 300 μl of Medium 199 containing EGM-2 without antibiotics was transferred into each well. The cells were then treated with a low (100 ng/ml) and high (1000 ng/ml) concentration of G129R (4.3 nM, 43 nM), endostatin (5 nM, 50 nM), or G129R-endostatin (2.4 nM, 24 nM). All assays were performed in triplicate and were repeated at least twice. Cells were incubated for 24–48 h at 37°C in a humidified 5% CO_2 incubator were and observed using a CK2 Olympus microscope (3.3 ocular, ×10 objective).

Pharmacokinetic Study. Female BALB/c mice (Jackson Lab, Bar Harbor, ME) were used to determine the serum-effective dose of G129R-endostatin after a single i.p injection. Two hundred μg of G129R (8.7 nmol), 200 μg of G129R-endostatin (4.8 nmol), or 200 μg (10 nmol) of endostatin was injected (i.p.) into BALB/c mice (n = 4). Blood samples were obtained from each mouse at time intervals of 2, 4, 8, and 24 h by tail vein bleeding. Samples were placed on ice and immediately centrifuged for 5 min at 4°C. The serum was collected and frozen at −20°C until further use. The serum concentration of both G129R and G129R-endostatin was determined using the hPRL IRMA kit (DPC, Inc.). Endostatin serum concentration was determined using the Accucyte ELISA method (Oncogene). Area under the curve (*AUC*) was calculated by linear trapezoidal method from 2 to 24 h.

Antitumor Effects *in Vivo*. The *in vivo* antitumor efficacy of G129R-endostatin was examined using a 4T1 mouse mammary xenograft in an athymic nude mouse model. Female athymic nude (*nu/nu*) mice (Jackson Lab) 6–8 weeks of age were randomly placed into groups of 5 mice/cage, two cages/treatment for a total of 10 mice/group. 4T1 breast cancer cells (5×10^4) were injected s.c. into the mammary fat pad of each mouse, and tumors were allowed to develop for 5 days. Once tumors were established, mice were subjected to daily i.p. injections of different agents as designed. Treatment groups were given G129R [2.5 mg (110 nmol)/kg/day], endostatin [2.5 mg (125 nmol)/kg/day], G129R-endostatin [5 mg (130 nmol)/kg/day], and a combination of G129R (2.5 mg/kg/day) and endostatin (2.5 mg/kg/day) in a volume of 100 μl. Control groups were given 100-μl injections of sterile PBS. Measurements of tumors were recorded every 5 days until it was decided that tumors were debilitating to the mice. The long axis (L) and the short axis (S) were measured, and the tumor volume (V) was calculated using the following equation:

$$V = \frac{S^2 \times L}{2}$$

Once final measurements were taken, the mice were sacrificed by cervical dislocation, and tumors were dissected, weighed, and flash-frozen and were stored in liquid nitrogen until analysis.

Statistical Analysis. The results from the MTS assay and the animal studies were presented as means ± SE (*error bars*). Statistical analysis was performed using the program StatsDirect, version 1.9.8 (CamCode, Cambridge, England) with one-way ANOVA and a Tukey-Multiple Comparison test.

RESULTS

Expression of G129R-Endostatin Fusion Protein. The recombinant fusion protein along with G129R and endostatin were purified from the inclusion bodies of *Escherichia coli* cells. As shown in Fig. 1*A*, all of the recombinant proteins migrate as a single band during SDS gel electrophoresis under reduced conditions in predicted sizes (G129R-endostatin, M_r 42,000; G129R, M_r 23,000; endostatin, M_r 20,000). Western blot analysis was used to further confirm the presence of both G129R and endostatin in the G129R-endostatin fusion protein (Fig. 1*B*).

G129R-Endostatin Binds to Both Human Breast Cancer and Endothelial Cells. The ability of G129R-endostatin to directly bind to the PRLR on the human breast cancer cell line T-47D was demonstrated using a radioreceptor binding assay (Fig. 2). It was determined that PRL, G129R, and G129R-endostatin all competitively displaced the ^{125}I-labeled hPRL from the PRLR on T-47D cells with similar affinity, whereas endostatin did not, suggesting that G129R-endostatin retained its ability to recognize PRLR.

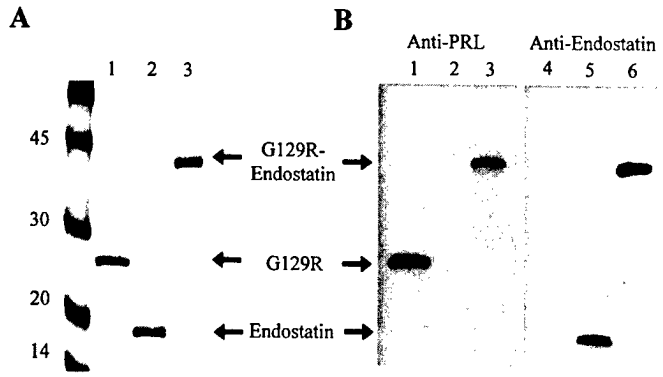

Fig. 1. Production and expression of G129R-endostatin. G129R and endostatin were cloned into the pET22b(+) expression vector. *A*, SDS-PAGE analysis of G129R-endostatin stained with Coomassie Blue. *At left*, molecular weight markers (kDa) in thousands. G129R migrates at M_r 23,000 (*Lane 1*) and endostatin at M_r ~20,000 (*Lane 2*). G129R-endostatin migrated at M_r ~43,000 (*Lane 3*). *B*, Western blot analysis of G129R-endostatin. *Lanes 1* and *4*, G129R; *Lanes 2* and *5*, endostatin; *Lanes 3* and *6*, G129R-endostatin. The left blot, *Lanes 1–3*, was incubated with a polyclonal rabbit anti-hPRL antibody and the right blot, *Lanes 4–6*, was incubated with a polyclonal rabbit antiendostatin antibody. A goat antirabbit IgG horseradish peroxidase conjugate was used as secondary antibody and was detected with ECL.

An immunofluorescence assay was used to determine whether G129R-endostatin can bind to both breast cancer and endothelial cells (Fig. 3). HUVEC and T-47D cells were treated with G129R, endostatin, or G129R-endostatin and were stained with protein-specific primary antibodies. Fluorescent secondary antibodies were used to distinguish G129R (Alexa Flour 594, Red) and endostatin (Alexa Flour 488, Green). Fig. 3, *A* and *B* represent the untreated HUVECs and T-47D cells as controls. As shown in Fig. 3, *C* and *D*, G129R-endostatin bound to HUVEC and T-47D cells, respectively. This is demonstrated by the fluorescence of both the endostatin antibody (*green*) and the PRL antibody (*red*) in the same field of view. Endostatin bound to HUVECs (Fig. 3*E*) and bound to what appears to be the ECM of T-47D cells with a scattered staining pattern (Fig. 3*F*). In contrast, G129R bound only to T-47D cells (Fig. 3*H*), but it did not bind to HUVECs (Fig. 3*G*). The distinct pattern of staining of G129R and endostatin is notable. G129R and G129R-endostatin treatments revealed a clear cellular staining pattern in T-47D cells (Fig. 3, *D* and *H*), whereas endostatin-treated cells demonstrated a scattered staining pattern in both HUVECs and T-47D cells (Fig. 3, *C*, *E*, and *F*). Because G129R did not bind to HUVECs caused by the lack of PRLR on these cells (Fig. 3*G*), the staining of HUVECs by G129R-endostatin (Fig. 3*C*) was most likely caused by the binding of the endostatin portion of the fusion protein.

G129R-Endostatin Inhibits STAT5 Phosphorylation in T-47D Human Breast Cancer Cells. STAT5 phosphorylation is one indicator of PRL-mediated signal transduction in mammary cells, and we have used this feature as a measure of the antagonistic effects of G129R and its variants (12). The status of STAT5 phosphorylation was examined after treatment of T-47D cells with PRL, G129R, endostatin, and G129R-endostatin. As shown in Fig. 4*A*, PRL (100 ng/ml, 4.3 nM) induced phosphorylation of STAT5 (pSTAT5), whereas G129R, endostatin, and G129R-endostatin, as expected, lacked the ability to induce STAT5 phosphorylation. A dose-dependent competitive inhibition of PRL-induced STAT5 phosphorylation was observed for G129R and G129R-endostatin (Fig. 4*B*). G129R and G129R-endostatin exhibited similar potency in the inhibition of

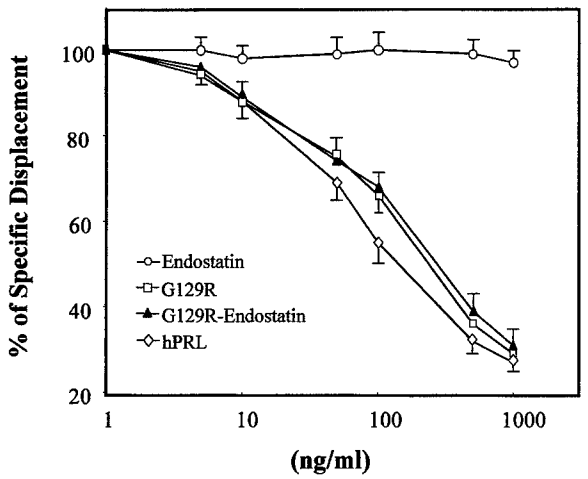

Fig. 2. Binding ability of G129R-endostatin to the PRL receptor on human breast cancer cells. The concentrations of the treatments are given on a log scale. The data are represented as the percentage of the displacement of ^{125}I-labeled hPRL (specific activity, 40 μCI/μg) compared with the total binding of each protein to human breast cancer cell line T-47D. The data represents the mean ± SD of three experiments.

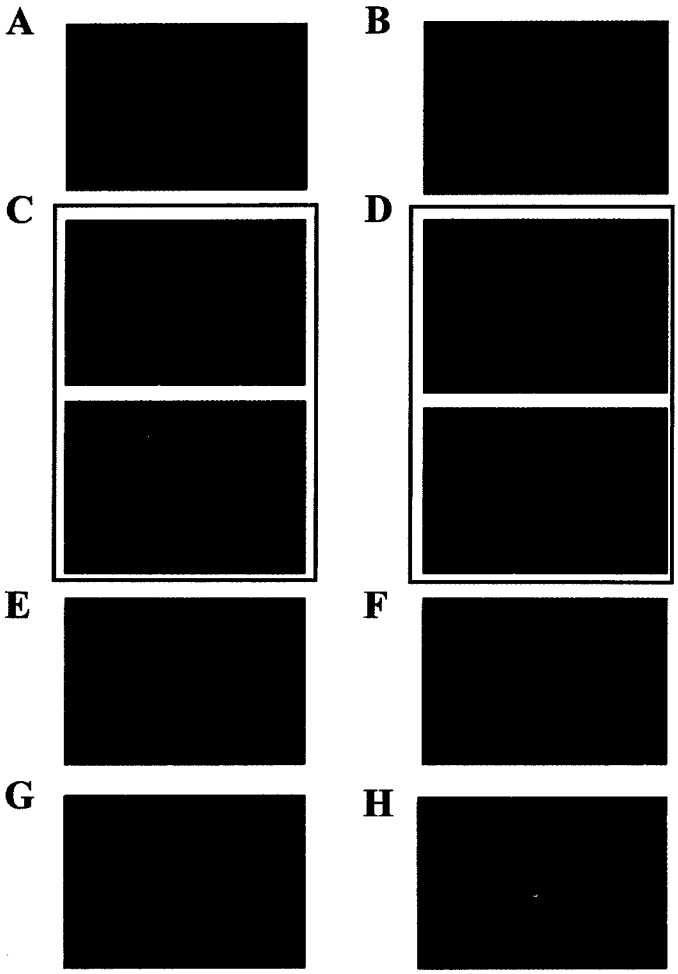

Fig. 3. Immunofluorescence staining of HUVECs and T-47D cells. *C* (HUVECs) and *D* (T-47D) represent cells treated with G129R-endostatin and stained with anti-hPRL and anti-human endostatin. *C* and *D* are boxed to represent the same field of view. Both *E* (HUVEC) and *F* (T-47D) represent cells treated with endostatin and G129R and stained with antihuman endostatin. Both HUVECs (*G*) and T-47D (*H*) cells were treated with endostatin and G129R and stained with anti-hPRL. Negative controls of HUVECs and T-47D cells were presented as *A* and *B*, respectively. The secondary antibodies used were Alexa Fluor 594 goat antimouse IgG (*red fluorescence,* PRL) and Alexa Fluor 488 goat antirabbit IgG (*green fluorescence,* endostatin), respectively, for each primary antibody. Digital photographs were taken at ×450.

STAT5 phosphorylation. This demonstrates that the G129R portion of G129R-endostatin retained its antagonistic effects toward PRLR.

G129R-Endostatin Inhibits the Proliferation of Human Endothelial and Human Breast Cancer Cells. Cell proliferation assays were carried out to examine the dual effects of G129R-endostatin in inhibiting the proliferation of both HUVECs and T-47D cells. G129R-endostatin was revealed to be as effective as endostatin in inhibiting the proliferation of HUVECs in a dose-dependent manner (Fig. 5*A*). The EC_{50} of G129R-endostatin (12 nM) was approximately one-half that of endostatin (25 nM; ~500 ng/ml; Fig. 5*A*). G129R had no effect on HUVEC proliferation, suggesting that the inhibitory effect of G129R-endostatin was caused by the endostatin domain of the fusion protein. Conversely, G129R-endostatin (EC_{50}, 18 nM) exhibited antiproliferative effects on T-47D human breast cancer cells similar to that of G129R (EC_{50}, 32 nM; ~750 ng/ml; Fig. 5*B*). As expected, endostatin had no effect on the proliferation of T-47D cells. Overall, G129R-endostatin was effective in inhibiting T-47D and HUVEC growth at molar concentrations much lower than those of G129R or endostatin, respectively.

G129R-Endostatin Fusion Protein Disrupts the Formation of Endothelial Tubes. An endothelial tube formation assay was used to further confirm the antiangiogenic activity of G129R-endostatin. In this experiment, the use of Matrigel permits the growth and differentiation of endothelial cells into tubal structures that are reminiscent of blood vessels. Prominent tubal structures were observed in the control cells (Fig. 6). At low concentrations (100 ng/ml; Fig. 6, *left column*) both endostatin and G129R-endostatin began to disrupt the formation of the tubes, indicated by the *arrows*. At high concentrations (1,000 ng/ml; Fig. 6, *right column*), both endostatin and G129R-endostatin treatments eliminated the tubal structures, and the cells appeared to be dying. G129R treatment, serving as a negative control in this experiment, had no obvious effects on endothelial tube formation.

Pharmacokinetic Comparison of G129R, Endostatin, and G129R-Endostatin Fusion Protein. It has been demonstrated that increasing the size of a protein may increase its half-life (29). The relatively short serum half-life of G129R and endostatin present a considerable challenge to the clinical use of these potential therapeutic agents. To examine whether the pharmacokinetics of G129R-endosta-

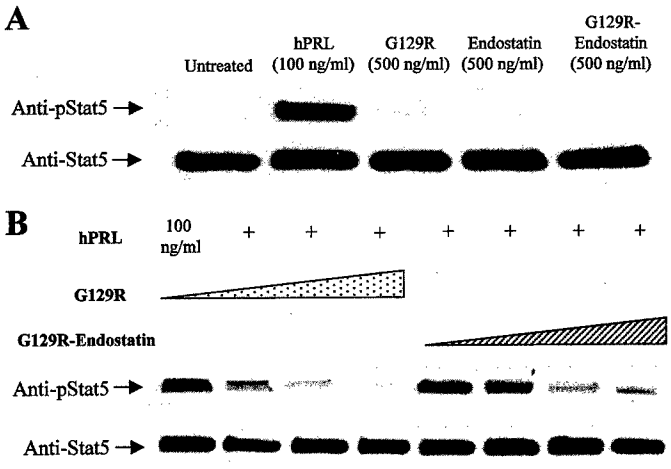

Fig. 4. Inhibition of STAT-5 phosphorylation by G129R-endostatin. T-47D human breast cancer cells were treated with the indicated amounts of PRL, G129R, and G129R-endostatin (*A*) or a dose-dependent combination treatment (*B*). Total protein was extracted and analyzed on a 4–15% gradient SDS-PAGE, followed by Western blotting with antiserum against either STAT-5-phosphorylated (*pStat5*) or STAT-5 as indicated in the appropriate panel. *A*, inhibition or stimulation of STAT5 phosphorylation of T-47D cells by PRL, G129R, endostatin, and G129R-endostatin. *B*, dose-dependent competitive inhibition of STAT-5-phosphorylation by G129R-endostatin. T-47D cells were incubated with PRL and increasing concentrations of G129R or G129R-endostatin. STAT5 and phosphorylated-STAT5 were detected by Western blot analysis as described in the "Materials and Methods."

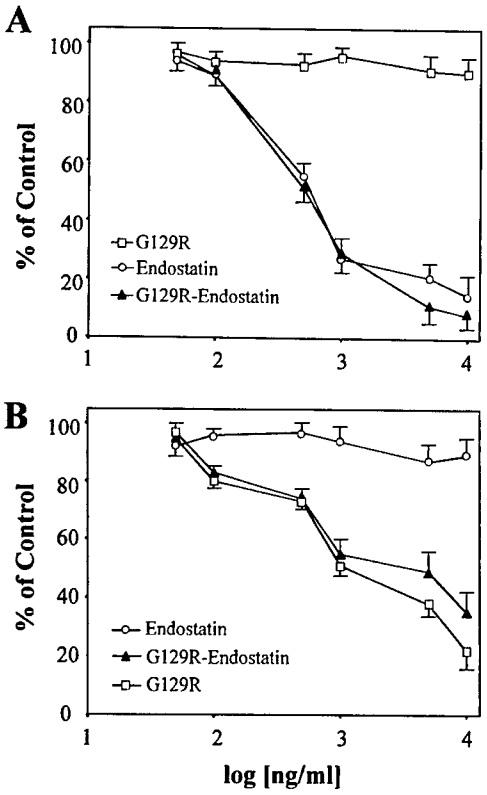

Fig. 5. Breast cancer and endothelial cell proliferation assay. Purified human endostatin, G129R-endostatin, and G129R were tested for their antiproliferative ability using HUVECs (*A*) and T-47D cells (*B*). Viability of cells was determined by the colorimetric MTS-PMS assay (Promega). Data are represented by the percentage of viable cells after treatments. *A*, ability of endostatin and G129R-endostatin to inhibit bFGF-induced endothelial cell proliferation using G129R as the control. *B*, effects of G129R and G129R-endostatin to inhibit the proliferation of human breast cancer cell line T-47D using endostatin as the control. Each experiment was carried out in triplicate, and the data are represented as the mean ± SE of three experiments.

tin were improved compared with G129R or endostatin alone, the *AUC* values of G129R, G129R-endostatin and endostatin were determined and compared after single i.p. injection (Fig. 7). The *AUC* was calculated for 200 μg of each protein based on the plot in Fig. 7. The *AUC* of G129R-endostatin is 7.3 times that of G129R and ~10 times that of endostatin. In addition, if taking into consideration the relative molar amounts of each protein injected (4.8 nmol of G129R-endostatin, as compared with 8.7 nmol of G129R or 10 nmol of endostatin was used in each experiment), the effective serum concentration of G129R-endostatin was found to be 13 times that of G129R and 21 times of equimolar amounts of endostatin. Thus, G129R-endostatin exhibits a higher effective serum concentration (and thus a longer serum half-life) than do G129R and endostatin.

G129R-Endostatin Fusion Protein Inhibits the Growth Rate of Breast Cancer Xenografts in Nude Mice. To test the efficacy of G129R-endostatin in inhibiting breast cancer, we used an aggressive murine breast cancer cell line, 4T1, in a nude mouse model. Fifty female athymic nude mice that were given injections of 4T1 cells (5×10^4) s.c. into the mammary fat pad were randomly divided into five groups. Postinoculation starting on day 5, G129R, endostatin, G129R-endostatin, and a combination treatment of G129R and endostatin were administered daily (i.p.). The control group was treated with 100 μl of PBS. Compared with control mouse group, all four of the treatments caused a significant reduction in tumor volume ($P < 0.0001$). Among the four treatment groups, G129R-endostatin (807 ± 235 mm^3) demonstrated the best inhibitory effects on 4T1 tumor growth and exhibited a statistically significant decrease in final tumor volume, compared with the control (2851 ± 305 mm^3; $P < 0.001$), G129R (1897 ± 194 mm^3; $P < 0.001$), endostatin (1271 ± 142 mm^3; $P < 0.001$), and the combination treatment (1399 ± 147 mm^3; $P = 0.0016$) groups (Fig. 8*A*). Similarly, all of the treatments caused significant reduction in the final tumor weights compared with the control group (1970 ± 410 mg): G129R-endostatin (841 ± 121 mg; $P < 0.001$); G129R (1409 ± 265 mg; $P < 0.001$), endostatin (1159 ± 170 mg; $P < 0.01$), and the combination of G129R and endostatin (1149 ± 195 mg; $P < 0.001$; Fig. 8*B*). G129R-endostatin treatment resulted in lower tumor weights than the other treatments in general. However, although this decrease was statistically significant compared with G129R ($P = 0.0004$), it was not significantly greater than that brought about by endostatin ($P = 0.0936$) or endostatin and G129R in combination ($P = 0.1065$).

DISCUSSION

Angiogenesis is the process of growth of new capillaries from preexisting blood vessels and is a crucial element for tumor sustenance (13). The switch of angiogenic phenotype in a tissue is dependent on the local balance between angiogenic factors and inhibitors (17). Of the many angiogenesis inhibitors that have been investigated and considered for potential cancer therapy, endostatin is one of the most potent and shows promise in inhibiting tumor growth in animals and in clinical trials (19).

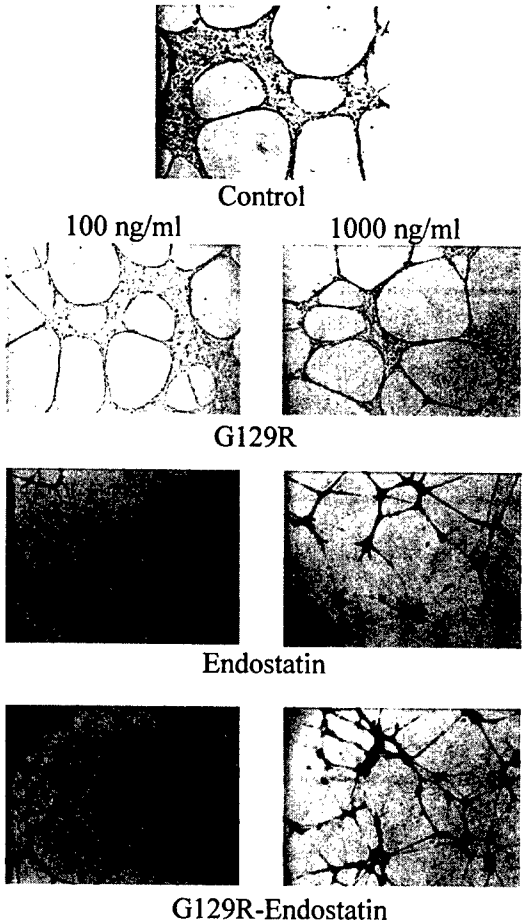

Fig. 6. The effect of G129R-endostatin on the three-dimensional structure of endothelial tubes. HUVECs (25,000 cells/well) in EGM-2 medium without antibiotic were plated onto Matrigel basement membrane-coated wells and were evaluated for their ability to form tubal structures similar to that of blood vessels. A low (100 ng/ml) and high (1000 ng/ml) concentration was used for endostatin, G129R-endostatin, and G129R. Each treatment was performed in triplicate. Untreated (*Control*) cells were processed similar to cells receiving drug treatment. Cells were viewed with a microscope and pictures were taken at ×10.

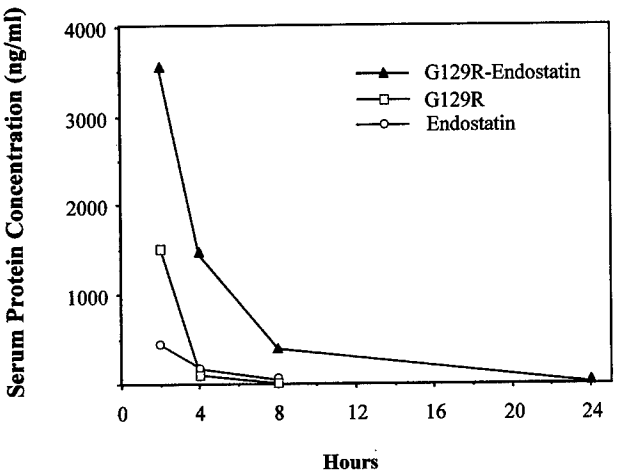

Fig. 7. Pharmacokinetic analysis of G129R-endostatin in BALB/c mice. Female BALB/c mice ($n = 4$) were given i.p. injections of G129R-endostatin (200 μg), G129R (200 μg), or endostatin (200 μg) and serum samples were collected by tail vein bleeding at the indicated time intervals. The serum concentration of G129R and G129R-endostatin was determined using the hPRL IRMA kit (DPC, Inc.). The serum concentration of endostatin was determined using the Accucyte ELISA protocol (Oncogene). The area under the curve (*AUC*) was determined for each protein. The *AUC* of G129R-endostatin is 7.3 times that of G129R. When adjusted for the relative molar amounts of each protein injected, the effective serum concentration of G129R-endostatin was found to be 13.1 times that of G129R for equimolar amounts of protein. The *AUC* for G129R-endostatin was 10-fold greater than the *AUC* for endostatin; this value was 21 for equimolar amounts of protein.

The underlying molecular mechanisms of antiangiogenic activity of endostatin are not fully understood, although several recent studies have begun to shed light on the mode of action of endostatin. Endostatin induces apoptosis causing G_1 arrest of endothelial cells through the inhibition of cyclin D1 (30) and may interrupt the Wnt signaling pathway, which is involved in cellular development (31). There is evidence that endostatin blocks the binding of vascular endothelial growth factor to endothelial cells (32) and inhibits the activation and catalytic activity of matrix metalloproteinases (33). Taken together, these studies suggest that the antitumor effects of endostatin are attributable to its specificity for endothelial cell proliferation rather than the direct inhibition of tumor cell growth (19). Successful attempts have been made to target endostatin to cancers of the breast and other tissues. For example, liposomes complexed with plasmids that encode endostatin inhibit breast tumor growth in mice when injected directly into tumors (34). Adenovirus-mediated systemic gene transfer of endostatin demonstrated significant reduction of tumor growth and inhibition of micrometastases in a mouse model (35). Together, these studies indicate that targeting endostatin directly to the tumor mass may improve the chance of tumor regression.

In view of the important role that PRL plays in breast cancer cell survival, the PRL antagonist, G129R, has demonstrated great potential as an antitumor agent. G129R inhibits breast cancer cell proliferation through the induction of apoptosis (10), in part, through the inhibition of *bcl-2* gene expression (13). Furthermore, G129R inhibits the growth of both T-47D and MCF-7 human breast cancer xenografts in nude mice (11). We have taken advantage of the ability of G129R to bind PRLR by designing targeted antitumor therapeutic agents. In this study, we genetically combined two proven effective anticancer agents that act via different mechanisms to create a novel bifunctional fusion protein, G129R-endostatin. We reasoned that a fusion protein consisting of G129R and endostatin would be targeted to breast cancer cells, inhibit tumor cell proliferation, and inhibit angiogenesis, which is required for proper development of the vascular network at the tumor site.

For endostatin to exert its antiangiogenic effects on the breast tumor microenvironment, both the G129R and the endostatin domains of G129R-endostatin fusion protein must recognize and bind receptors on breast cancer cells and endothelial cells, respectively. The specific binding of G129R-endostatin to the PRLR on breast cancer cells and to HUVECs was demonstrated by a radioreceptor binding assay and immunofluorescence/confocal microscopy. The binding affinity of G129R-endostatin to PRLR was similar to that of PRL and G129R. Thus, each portion of the fusion retained the ability to recognize its cognate receptor. The dual binding ability of the fusion protein was illustrated by dual immunofluorescence staining of both G129R and endostatin portions of G129R-endostatin. The binding pattern of endostatin to what appears to be the ECM in cultures of T-47D cells is interesting. The precise receptors/ligands to which endostatin binds have not been fully determined, and it is possible that, in the absence of preferred cell surface receptors on T-47D cells, endostatin associates with one or more ECM proteins. Because G129R itself has a high affinity for T-47D breast cancer cells, the G129R-endostatin fusion protein binds preferentially to these cells. Although the fusion protein binds to both breast cancer cells (T-47D) and endothelial cells via the appropriate domains, the individual domains of the fusion protein may not necessarily exhibit similar affinities for their respective ligands; the affinity of G129R for the PRLR may be greater than that of endostatin for its ligand(s) in the ECM. This may prove to be important in future clinical applications in which preferential localization

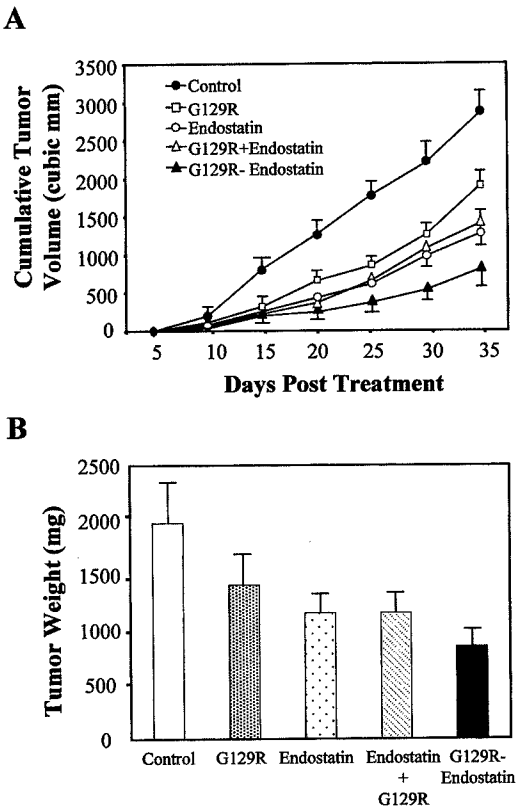

Fig. 8. *In vivo* analysis of human breast cancer inhibition using G129R-endostatin. Fifty athymic nude mice per group were inoculated (s.c.) with 5×10^4 4T1 cells. Tumors were allowed to establish for 5 days. Mice were randomized into five groups of 10 and were given injections of G129R (2.5 mg/kg/mouse), endostatin (2.5 mg/kg/mouse), G129R-endostatin (5 mg/kg/mouse), the combination of G129R (2.5 mg/kg/mouse) and endostatin (2.5 mg/kg/mouse), or 100 μl of sterile PBS for 35 consecutive days. *A*, tumor volume was determined every 5 days posttreatment by measuring the short axis (*S*) and the long axis (*L*) of the tumors and was calculated using the equation: $[S^2 \times L]/2$. Treatments of G129R-endostatin, G129R, endostatin, and G129R and endostatin in combination caused significant tumor reduction compared with the control group ($P < 0.005$). *B*, once the final tumor volume was measured, the tumors were removed and weighed. Values are represented as mean ± SE for each group ($n = 10$). All of the treatments caused significant reduction in the final tumor weights compared with the control group: G129R-endostatin ($P < 0.001$); G129R ($P < 0.001$), endostatin ($P < 0.01$), and the combination of G129R and endostatin ($P < 0.001$; *B*). In addition, G129R-endostatin-induced decrease in tumor weight was significantly greater than in G129R-treated mice ($P = 0.0004$), but not the endostatin ($P = 0.0936$) or endostatin and G129R combination ($P = 0.1065$) groups.

of G129R-endostatin to breast tumor tissue, instead of to vascular tissue in general, is essential.

Drug efficacy is, in part, affected by its serum half-life, a property that can be improved by increasing the size of a given molecule or protein (36). A potential limitation of the use of G129R and endostatin in cancer treatment is their relatively short serum-half-lives (29). One incentive to generate G129R-based fusion proteins for cancer therapy was to increase the serum half-life of G129R by increasing its size, a strategy that we used to generate a G129R fusion with interleukin 2 (G129R-interleukin 2; 29). In previous studies, G129R inhibited breast cancer xenografts at a dose of 5 mg (220 nmol)/kg/day (11), whereas inhibition of tumor growth, and an increase in serum half-life could be achieved by increasing endostatin to 20 mg (1 μmol)/kg/day (19, 21). The serum half-life of endostatin in mice has been found to be ~5 h (37). We have increased the serum half-life of G129R by mixing it with Matrigel or incorporating it into slow-releasing pellets (11), however, these methods currently are unsuitable for clinical studies. In this study, we demonstrate that one advantage of generating novel fusion proteins as therapeutics is that, along with the increased molecular size of the fusion protein, their serum half-lives are usually greatly extended. The effective serum concentration of G129R-endostatin is maintained over 24 h as shown in Fig. 7; this is significantly longer than that of either endostatin or G129R. We believe that this feature should contribute significantly in enhancing the antitumor effects of G129R-endostatin, especially in a clinical setting.

In summary, we have created a novel fusion protein, G129R-endostatin, consisting of the PRL antagonist G129R and the antiangiogenic protein endostatin. The fusion protein is a bifunctional protein that exhibits characteristics of G129R (the inhibition of breast cancer cell proliferation) and endostatin (inhibition of endothelial cell proliferation and development). More importantly, G129R-endostatin inhibits tumor growth at a dose much lower (5 mg/kg/day) than that reported for previous endostatin treatments (20 mg/kg/day). Given its bifunctional nature, G129R-endostatin, could become a potential therapeutic agent for the treatment of human breast cancer. Additional studies of the *in vivo* efficacy of G129R-endostatin will support its potential benefit in clinical application. The shortcomings of endostatin Phase II/III clinical trials may be ameliorated by a strategy, as described here, which increases the effective serum concentration of endostatin and targets it directly to the tumor site.

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Inhibition of oncogene STAT3 phosphorylation by a prolactin antagonist, hPRL-G129R, in T-47D human breast cancer cells

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Abstract. We have previously demonstrated that a hPRL antagonist (hPRL-G129R) was able to inhibit PRL induced breast cancer cell proliferation through induction of apoptosis. In the present study, we test the hypothesis that the inhibitory effect of hPRL-G129R in breast cancer cells occurs, at least in part, through the inhibition of oncogene STAT3 activation. We first demonstrated that STAT5 and STAT3 could be activated by either hGH or hPRL in T-47D breast cancer cells. Although the patterns of STAT5 activation by hGH and hPRL are similar, we observed a nearly 10-fold greater efficacy of hPRL in STAT3 activation as compared to that of hGH. More importantly, we have demonstrated that activation of STAT3 by hPRL could be inhibited by hPRL-G129R. Since T-47D cells coexpress GHR and PRLR, an attempt was made to dissect the molecular events mediated through hGHR or hPRLR using mouse L-cells expressing a single population of receptors (hGHR or hPRLR). To our surprise, only STAT5, not STAT3 phosphorylation was observed in these L-cells. In conclusion, our results suggest that: a) STAT3 is preferably activated through hPRLR in T-47D cells; b) hPRL-G129R is effective in inhibiting STAT3 phosphorylation; and c) the mechanism of STAT3 activation is different from that of STAT5.

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Abbreviations: hPRL, human prolactin; hGH, human growth hormone; hPRL-G129R, human prolactin antagonist; PRLR, prolactin receptor; GHR, growth hormone receptor; STAT, signal transducer and activator of transcription; Cys, cysteine; L-GHR, mouse L-cells expressing GHR; L-PRLR, mouse L-cells expressing PRLR; E2, estradiol; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; bGH, bovine GH; FBS, fetal bovine serum; CSS, charcoal stripped serum; IPTG, isopropyl-thiogalactoside; IRMA, immunoradiometric assay; MET, methionine

Key words: prolactin antagonist, breast cancer, STAT3

Introduction

STAT (signal transducers and activators of transcription) proteins are important transcriptional regulators in the cell, and have been studied in great detail (1-6). Seven STAT genes have been identified that encode eight different STATs; STAT1α, 1ß, 2, 3, 4, 5a, 5b and 6 (1,2,4,7). Each STAT plays an important, yet different role in signal transduction. STAT proteins have two main functions that include signal transduction in the cytoplasm and activation of transcription in the nucleus (2,4). STATs are usually activated in response to a ligand/receptor interaction. Binding of cytokines or hormones to their respective receptors stimulates the Janus kinase family of proteins which then phosphorylate STAT proteins on a specific tyrosine residue at the COOH terminus (4). Homo- or heterodimers are formed between the phosphorylated tyrosine of one STAT molecule and the SH2 domain of another STAT molecule. These dimers translocate into the nucleus, by a mechanism that is unknown, and function as transcription factors by binding to their recognition sequences and regulating the target gene expression (3-5).

STAT5 and STAT3 have been shown to be critical in mammary gland development by homologous recombination gene disruption studies in mice (8,9). Given the importance of STATs in the control of mammary gland developmental processes and their intimate association with cytokines and hormones, it is not surprising that inappropriate activation of STATs has been found in human breast cancer and other malignancies (10-12). The autocrine/paracrine effects of certain ligands including PRL or GH have been reported to increase activity of tyrosine kinases and therefore the hyperactivity of STATs (13-16). STAT3, which was initially identified in interleukin 6 induced signaling pathways (17,18), recently was shown to be significant in cancer. This is based on the finding that certain forms of cancer and tumor cell lines show constitutively active STAT3 (19-24) and that STAT3 can transform cells (25-29). In addition, a naturally occurring mutant form of STAT3, termed STAT3ß, was found to be able to suppress the growth of B16 melanoma cells *in vitro* and *in vivo* (30). STAT3ß has a mutation in the carboxy region of STAT3, and therefore it is not able to activate transcription. More recently, several lines of evidence clearly elucidate the functional role of STAT3 as an oncogene (31). A constitutively

active form of STAT3 was developed in which amino acid residues important for STAT3 dimerization were replaced with Cys. This Cys substitution resulted in a constitutively dimerized (via homodimerization through disulfide bonds) and therefore active form of STAT3. The mutated form of STAT3 had the ability to transform cells and to induce tumor formation *in vivo*. It was also shown that constitutively active STAT3 leads to increased c-*myc* and cyclin mRNA which is important for cell proliferation (32-34) and increased Bcl-X_L mRNA, which is an anti-apoptotic factor (35,36). The above lines of evidence point to the possibility of using STAT3 as a therapeutic target.

There is a high incidence of breast cancer in women from Western countries, but the cause of breast is still unknown. Recently, the relationship between hPRL and breast cancer has been re-emphasized (37-41). After the finding of locally produced PRL by the mammary gland and the up-regulation of PRLR in breast cancer samples (37,40,41), hPRL is now considered as an autocrine/paracrine growth factor that contributes to breast cancer development. It is believed that local production of PRL by breast cancer cells results in autocrine/paracrine stimulation of PRL receptors that perhaps leads to uncontrolled cell proliferation (14,16). STATs 3 and 5 are involved in PRL mediated signal transduction (7), therefore suggesting a role for these factors in breast cancer.

In our recent studies, we have demonstrated that a single amino acid substitution at position 129 of hPRL (hPRL-G129R) resulted in a true hPRL receptor antagonist in human breast cancer cell based assays (42). We have shown that: a) hPRL and E2 exhibit additive stimulatory effects on human breast cancer cell proliferation, suggesting that these two stimuli act together through different mechanisms to promote cell proliferation; b) hPRL-G129R binds to the hPRLR with an affinity similar to that of wild-type hPRL; c) hPRL-G129R inhibited breast cancer cell proliferation; and d) when anti-estrogen (4-OH-tamoxifen) and anti-PRL (hPRL-G129R) agents were applied simultaneously, there was an additive inhibitory effect (42). We further investigated the mechanism of the inhibitory effects of hPRL-G129R. Using multiple human breast cancer cell lines, we also demonstrated that hPRL-G129R was able to induce apoptosis in a dose dependent manner as determined by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. The main goal of our current study is to further elucidate the role of hPRL in breast cancer, in particular the relationship between hPRL, STAT5 and STAT3. In view of the fact that STAT3 has been shown to be an oncogene, we are especially interested to see if hPRL-G129R is able to inhibit the activation of STAT3 in human breast cancer cells.

Materials and methods

Cell lines used for STAT3 and STAT5 phosphorylation studies. T-47D cells, a human breast cancer cell line, were obtained from the American Type Culture Collection (ATCC, Rockville, MD). They were maintained in phenol red-free RPMI 1640 media supplemented with 10% fetal bovine serum (Life Technologies Inc.). L-GHR cells and L-PRLR cells were established by extracting mRNA from T-47D breast cancer cells using the Micro-Fast Track 2.0 kit available from Invitrogen Corp. (Carlsbad, CA). The full-length cDNA encoding hPRLR and hGHR were cloned using RT-PCR (using a RT-PCR kit from Promega Corp.). Full-length hPRLR and hGHR cDNAs were then cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) and sequences were confirmed using an ABI 310 Genetic Analyzer. Both cDNA sequences were identical to that published in GeneBank (data not shown). The hGHR and hPRLR cDNAs were then sub-cloned into a pIG-Met expression vector containing the mouse metallothionein regulatory sequences and bGH polyA signal. This expression vector has been used in many of our previous studies (43-45).

TK (thymidine kinase) and APRT (adenine phosphoribosyl transferase) mouse L-cells were used to establish stably transfected hGHR and hPRLR L-cells as described previously (44,45). Briefly, L-cells were transfected with the plasmids using lipofectin (Life Technologies). HAT (hypoxathine aminopterin thymine) resistant colonies were isolated and propagated in tissue culture flasks. Positive L-hGHR and L-hPRLR cells were identified by RT-PCR and subsequently verified by receptor binding assay (data not shown). The stable cell lines with high expression levels were then propagated and maintained in DMEM supplemented with 10% FBS (Life Technologies Inc.). All cell lines were grown at 37°C in an atmosphere containing 5% CO_2.

E. coli production and purification of hPRL-G129R. hGH and hPRL used in this study were a kind gift from Dr A.F. Parlow (National Hormone and Pituitary Program, NIH). The hPRL-G129R used in this study was produced from *E. coli* according to published protocols (46,47) with modifications. Briefly, BL21(DE3) cells (Novagen, Madison, WI) were transformed with hPRL-G129R plasmid using the calcium chloride method. The transformant was spread on an ampicillin plate, and grown overnight at 37°C. The LB seed culture was inoculated with 6-10 colonies and grown overnight. The following day an LB growth culture was generated by inoculation of 5% of the seed culture and grown for ~2.5 h at 37°C with agitation. IPTG (Fisher Scientific) was then added to the culture (1 mM final concentration) to induce expression of hPRL-G129R and incubated for an additional 4 h. Bacteria were pelleted and resuspended in a solution containing 0.2 M $NaPO_4$ pH 8.0, 10 mM EDTA, and 0.5% Triton X-100. The resuspended bacteria were lysed with a 550 Sonic Dismembrator (Fisher Scientific). The hPRL-G129R product, which is in the form of an inclusion body, was pelleted at 12,000 g for 15 min and resuspended in 0.2 M $NaPO_4$ pH 7.0, 1% v/v ß-mercaptoethanol, 8 M urea for refolding. The refolding process consisted of dialyzing the protein against decreasing amounts of urea and ß-mercaptoethanol in the presence of 50 mM NH_4HCO_3 pH 8.0 for three consecutive days. The sample was first filtered through a 0.22 micron filter (VWR), degassed and then purified by a Q-Sepharose anionic exchange column (Pharmacia, Piscataway, NJ) using a FPLC system (Pharmacia, Piscataway, NJ). The concentration of hPRL-G129R purified from FPLC was determined using the Prolactin IRMA kit (DPC, Los Angeles, CA). The purity of hPRL-G129R was over 90% on SDS-PAGE using the silver staining method (Biorad, Hercules, CA). The hPRL-G129R produced by this

method has an extra Met at the N-terminus as compared to wild-type PRL.

Extraction of protein from cultured cells for STAT3 and STAT5 assays. Twenty-four hours prior to protein extraction T-47D cells were resuspended in RPMI media containing 10% charcoal stripped serum (CSS; Hyclone, Logan, UT), plated into 6-well plates and grown to confluency. L-cells expressing either GHR or PRLR were resuspended in DMEM containing 10% FBS and plated into 6-well plates to confluency. On the day of treatment, T-47D cells were depleted for 30 min in RPMI containing 0.5% CSS and L-cells expressing either GHR or PRLR were depleted for 2 h in DMEM. Cells were treated for 20 min with the appropriate amount of hGH (NIH), hPRL (NIH, National Hormone and Pituitary Program) or hPRL-G129R (produced in our laboratory). Cells were then washed with ice cold PBS (Life Technologies) and 200 µl of lysis buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EGTA; 1 mM PMSF; 1 µg/ml aprotinin and 1 µg/ml leupeptin; and 1 mM Na_3VO_4) was added to each well. Cells were incubated on an orbital rotator for 15 min and then lysate was transferred to a 1.5 ml Eppendorf tube. Lysate was gently passed through a 21 gauge needle 5-6X to shear genomic DNA and then placed on ice 20 min. Lysate was spun at 14,000 rpm for 20 min at 4°C in a microcentrifuge.

Preparation of cell lysates for STAT3 and STAT5 analysis. Thirty-five µl of cell lysate (65-70 µg) was added to 15 µl of 3X SDS-PAGE sample buffer. Total protein obtained from cultured cells was approximately equal for all cell lines used as determined by the Bradford protein assay (Biorad, Hercules, CA). Samples were heated for 5 min at 100°C, and then analyzed on a 4-15% gradient gel (Biorad, Hercules, CA). Protein was transferred to Hybond nitrocellulose membrane (Amersham, Arlington Heights, IL) for 2.5 h at 12 W. A high molecular weight rainbow marker (Amersham, Arlington Heights, IL) was used to determine the protein size as well as the success of the transfer.

Analysis of STAT3 and STAT5 protein levels. The protocol used to determine STAT3 levels or STAT5 levels was obtained from Upstate Biotechnology Institute (UBI, Lake Placid, NY). The protocol was altered slightly, and is described below. After protein transfer, membranes were washed briefly with distilled water and then blocked for 20 min in PBS containing 3% non-fat powdered milk (Biorad, Hercules, CA) for STAT3 analysis and in TBS containing 5% non-fat powdered milk, and 0.05% Tween-20 for STAT5 analysis. Membranes were then incubated in either STAT3 antiserum (UBI, Lake Placid, NY) at a concentration of 2 µg/ml or a 1:4,000 dilution of STAT5a antiserum and a 1:4,000 dilution of STAT5b antiserum (UBI, Lake Placid, NY) overnight at 4°C with constant agitation. Membranes were washed twice with distilled water (5 min/wash), and were incubated in a 1:2,000 dilution of goat anti-rabbit horseradish peroxidase secondary antibody (Biorad, Hercules, CA) for 2 h at room temperature with constant agitation. After secondary antibody incubation, membranes were washed once with distilled water, once with PBS containing 0.05% Tween-20, and once with distilled water. Membranes were developed for 1 min using enhanced chemiluminescence reagents (ECL; Amersham, Arlington Heights, IL). Membranes were then exposed to Kodak MR film (Fisher).

STAT3 and STAT5 tyrosine phosphorylation analysis. The same overall protocol was followed as for the analysis of STAT3 and STAT5 total protein levels. Membranes were incubated overnight with constant agitation in mouse phospho-STAT3 antiserum (UBI, Lake Placid, NY) at a concentration of 1.7 µg/ml or in mouse phospho-STAT5a/b antiserum (UBI, Lake Placid, NY) at a concentration of 1.5 µg/ml. Phospho-STAT3 antiserum was specific for phosphorylated tyrosine 704, and phospho-STAT5 antiserum was specific for phosphorylated tyrosine 694 for STAT5a and 699 for STAT5b. After primary antibody incubation, membranes were incubated in a 1:2,000 dilution of goat anti-mouse horseradish peroxidase conjugated secondary antibody (Biorad, Hercules, CA) for 2 h at room temperature. Membranes were washed as described above and developed for 1 min using ECL reagents. Membranes were exposed to Kodak MR film.

Results

Dose response studies for STAT5 and STAT3 phosphorylation in T-47D breast cancer cells. Dose response studies for STAT3 and STAT5 phosphorylation were first carried out in T-47D cells, which coexpress hGHR and hPRLR. T-47D cells were treated with increasing concentrations of either hPRL or hGH. It is clear that STAT5 can be maximally activated by either hPRL or hGH in T-47D cells at a dose of approximately 250 ng/ml (Fig. 1). Activation of STAT3 was also observed in T-47D cells treated with either hPRL or hGH (Fig. 2). However, the levels of STAT3 phosphorylation induced by hPRL at a concentration of 50-100 ng/ml were compatible to the levels of STAT3 phosphorylation induced by hGH at 500-1,000 ng/ml (Fig. 2).

hPRL-G129R inhibits STAT3 phosphorylation induced by hPRL in T-47D cells. hPRL-G129R inhibits hPRL induced STAT3 phosphorylation in T-47D cells. Fig. 3 shows the results of competition studies in which T-47D cells were treated with hPRL, hPRL-G129R, or a combination of the two in different concentrations. It is clear that hPRL-G129R is not active in terms of STAT phosphorylation (either STAT5 or STAT3; Fig. 3). At a 1:1 ratio of hPRL-G129R:hPRL, phosphorylation of STAT3 was significantly inhibited, whereas at a 5:1 ratio, phosphorylation of STAT3 is completely inhibited (Fig. 3b). hPRL-G129R appears to inhibit STAT3 phosphorylation to a greater extent than STAT5 phosphorylation (Fig. 3a).

STAT5, but not STAT3, is activated in L-GHR or L-PRLR cells. Because T-47D cells coexpress PRLR and GHR, we wanted to look at STAT3 and STAT5 phosphorylation in the presence of a single population of receptors using L-GHR or L-PRLR cells. STAT5 phosphorylation was detected at very high levels in both L-GHR or L-PRLR cells [there is no activation in parental L-cells as described previously (48) suggesting the activation is through transfected human

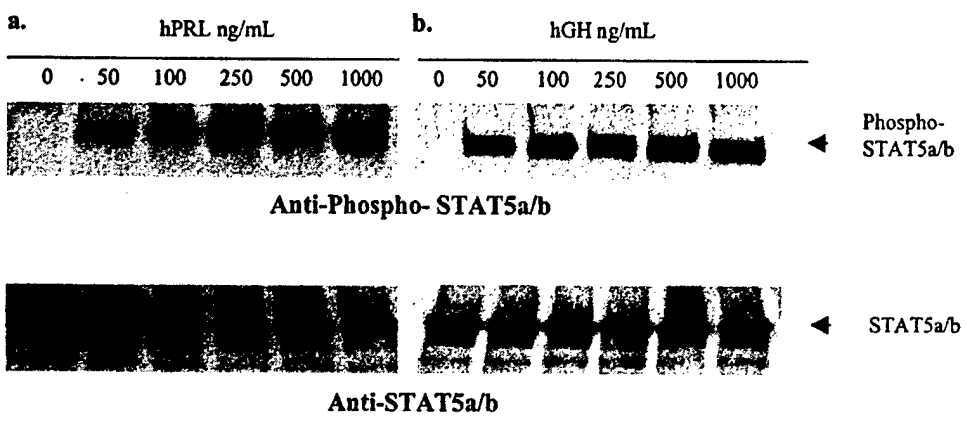

Figure 1. Dose response studies for STAT5 phosphorylation in T-47D cells. Cells were treated with increasing amounts of either hPRL or hGH. Total cellular protein was extracted from cells and subject for gradient SDS-PAGE followed by Western analysis with phospho-STAT5 antiserum (a and b, top) and anti-STAT5a and STAT5b antiserum (a and b, bottom).

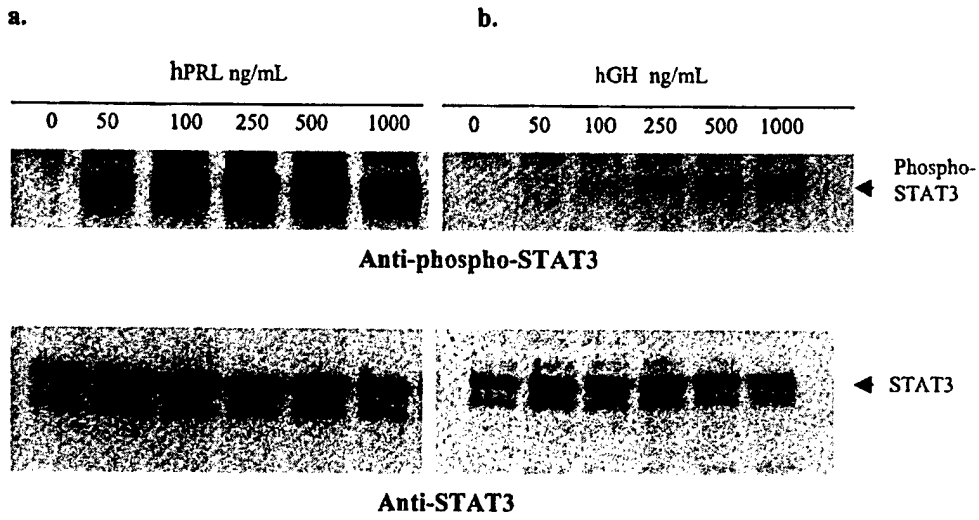

Figure 2. Dose response studies for STAT3 phosphorylation in T-47D cells. Cells were treated with increasing amounts of either hPRL or hGH. Total cellular protein was extracted from cells and subject for gradient SDS-PAGE followed by Western analysis with phospho-STAT3 antiserum (a and b, top) and anti-STAT3 antiserum (a and b, bottom).

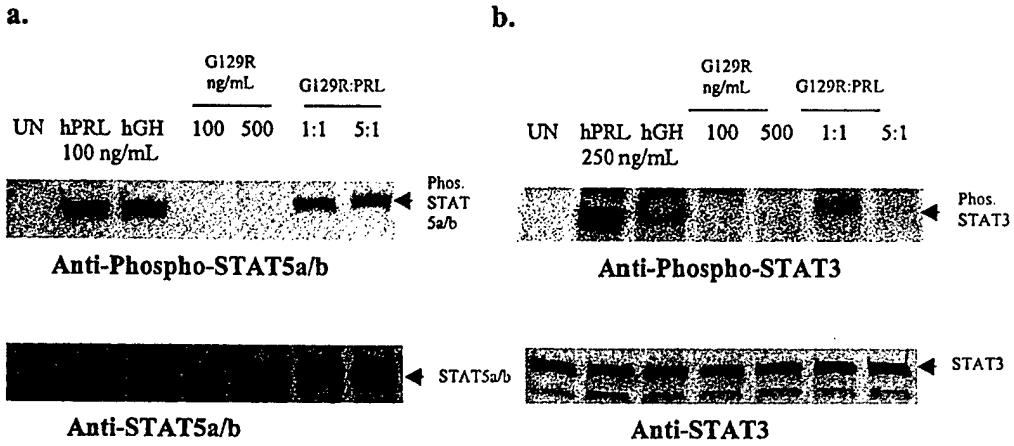

Figure 3. Inhibition of hPRL induced STATs phosphorylation by hPRL-G129R in T-47D cells. Cells were treated for 20 min with hPRL, hGH, or hPRL + hPRL-G129R. The amount of hPRL used for STAT5 competition studies was 100 ng/ml whereas 250 ng/ml was used for STAT3 studies since at these concentrations the phosphorylation of STATs reached maximal level. The untreated (UN) cells were used as a control. A 1:1 ratio or a 5:1 ratio of hPRL-G129R:hPRL was used for treatment. Total cellular protein was then extracted from cells and analyzed by Western blotting with either phospho-STAT5a/b antiserum (a, top) or phospho-STAT3 antiserum (b, top) or STAT5 antiserum (a, bottom) or STAT3 antiserum (b, bottom).

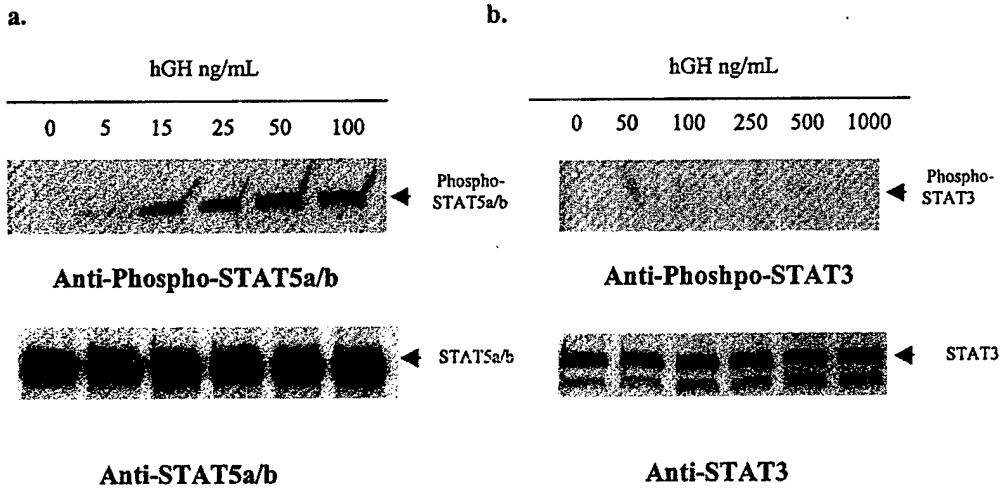

Figure 4. Dose response studies for STAT5 and STAT3 phosphorylation in L-GHR cells. Cells were treated with increasing amounts of hGH and then total protein was extracted. Maximum STAT5 phosphorylation is detected in L-GHR cells at a concentration of 50 ng/ml of hGH (a, top). STAT3 phosphorylation was not detected in L-GHR cells treated with hGH (b, top). Protein levels were equal in each case as indicated by Western analysis with either STAT3 antiserum or STAT5a/b antiserum (a and b, bottom).

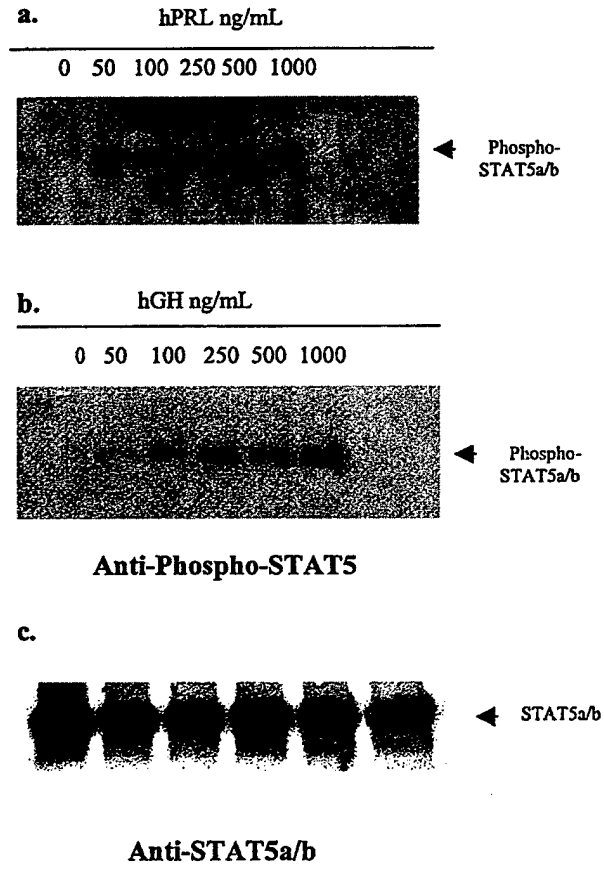

Figure 5. Dose response studies for STAT5 phosphorylation in L-PRLR cells. L-PRLR cells were treated with increasing amounts of hPRL and hGH. The cellular protein was then extracted. Protein was analyzed by Western blotting with either phospho-STAT5 antiserum (a and b) or antiserum against STAT5 protein (c). Maximum STAT5 phosphorylation is seen at a hPRL concentration of 250 ng/ml (a, top). Maximum STAT5 phosphorylation is seen at a hGH concentration of 1,000 ng/ml (b, middle). Panel c showing that an equal amount of protein was loaded in each well. This panel is representative of cells treated with either hPRL or hGH.

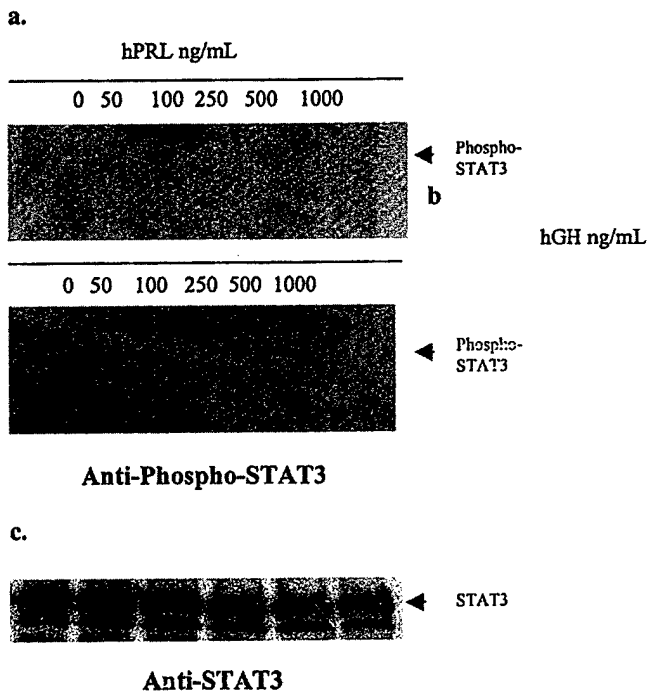

Figure 6. Dose response studies for STAT3 phosphorylation in L-PRLR cells. L-PRLR cells were treated with increasing amounts of hPRL and hGH. The cellular protein was extracted. Protein was analyzed by Western blotting with either phospho-STAT3 antiserum (a and b) or antiserum against STAT3 protein (c). STAT3 phosphorylation is not detected in L-PRLR cells either treated with hPRL (a, top) or hGH (b, middle). Equal amounts of STAT3 protein were seen at a relatively high level in each lane (c, bottom). Panel c is representative of cells treated with either hPRL or hGH.

receptors; Figs. 4a, 5a and b]. 50 ng/ml of hGH is able to induce maximum phosphorylation of STAT5 in L-GHR cells (Fig. 4a). On the other hand, STAT5 phosphorylation was observed in L-PRLR cells when these cells were stimulated by either hPRL or hGH at the compatible concentration range (Fig. 5a and b). Interestingly, the concentration required for hGH to induce maximum STAT5 phosphorylation in L-PRLR cells is much higher as compared to that needed in L-GHR cells (Figs. 4a and 5b). As expected,

STAT5 phosphorylation was not observed in L-GHR cells treated with hPRL, since hPRL does not bind to hGHR (data not shown).

To our surprise, when L-GHR or L-PRLR cells were treated with hGH or hPRL, STAT3 phosphorylation was not detected (Figs. 4b, 6a and b) despite the fact that relatively high levels of STAT3 protein are present (Figs. 4b and 6c).

Discussion

The role of hPRL in human breast cancer has recently been re-emphasized (37-41). In our previous studies, we demonstrated that a hPRL antagonist with a single amino acid substitution, hPRL-G129R, was able to inhibit hPRL induced human breast cancer cell proliferation through induction of apoptosis (42). In this study, we tested the hypothesis that the inhibitory effects of hPRL-G129R on human breast cancer cells are mediated, at least in part, through the inhibition of STAT phosphorylation, and in particular, STAT3 phosphorylation. The results from T-47D breast cancer cells demonstrated that both STAT5 and STAT3 are tyrosine phosphorylated in response to either PRL or GH (Figs. 1 and 2). A similar pattern of STAT5 activation induced by hGH or hPRL was observed (Fig. 1). However, hPRL is much more efficient in activating oncogene STAT3 as compared to that of hGH in T-47D cells (Fig. 2). There is approximately a 10-fold difference between these two ligands (the levels of STAT3 phosphorylation induced by 50-100 ng/ml of hPRL are equivalent to that induced by 500-1,000 ng/ml of hGH, Fig. 2). Although lacking direct evidence, we speculate that the activation of STAT3 by hGH is probably due to the fact that hGH is able to bind to hPRLR. The difference in efficacy between hGH and hPRL in inducing STAT3 phosphorylation probably reflects the difference between a homologous system (hPRL/hPRLR interaction) and a heterologous system (hGH/hPRLR interaction).

We further demonstrated that hPRL-G129R is able to competitively inhibit STAT3 phosphorylation induced by hPRL in T-47D cells (Fig. 3). At a 5:1 ratio (hPRL-G129R: hPRL), hPRL-G129R can completely inhibit STAT3 phosphorylation. It is of interest that inhibition of STAT3 phosphorylation by hPRL-G129R is much more efficient as compared to its ability to inhibit STAT5 phosphorylation (Fig. 3). This data further strengthens our speculation that STAT3 activation is more specific to the hPRLR pathway.

It is known that T-47D cells coexpress GHR and PRLR. In an attempt to differentiate the STAT phosphorylation events mediated through hGHR or hPRLR, we cloned full length hGHR and hPRLR cDNA from T-47D cells and established mouse L-cells with a single population of either hGHR or hPRLR. As expected, STAT5 activation was observed in both cell lines when exposed to respective ligands (Figs. 4a and 5a). As expected hGH is able to activate STAT5 in L-hPRLR cells but hPRL was inactive in L-hGHR cells. It is worthy to point out that the concentration needed for hGH to elicit strong STAT5 phosphorylation in L-PRLR cells is much higher than that in L-GHR cells. The data provide further evidence that a heterologous ligand/receptor interaction is less efficient than a homologous ligand/receptor system. To our surprise, however, we did not observe any STAT3 phosphorylation event in either of the cell lines even at the highest ligand concentration (Figs. 4b, 6a and b) despite the fact that relatively high amounts of STAT3 protein are present in these L-cells (Figs. 4b and 6c). One explanation for this obvious difference between the activation of STAT5 and STAT3 in these stable L-cell lines is that the crucial factors that link the events between ligand/receptor activation and STAT phosphorylation are unique for individual STAT activation. The factors that link ligand/receptor activation to STAT5 phosphorylation are common or can be shared between human cells (T-47D) and mouse L-cells. However, the factors that link ligand/receptor activation to STAT3 phosphorylation are either species specific or are missing or mutated in L-cells. It is also necessary to determine if the activation of STATs seen in T-47D breast cancer cells, but not in fibroblast mouse L-cells, is a breast cancer cell specific phenomenon. If this is true, the status of STAT3 phosphorylation might provide a clinical indication of the application of the hPRL antagonist.

In conclusion, cancer is a disease in which one of the hallmarks is uncontrolled cell proliferation and aberrant signal transduction (49,50). For example, cancer cells may overexpress a specific signal transduction factor; such is the case in the constitutive activation of oncogene STAT3 (31). It is possible that the constant presence of hPRL (an autocrine/paracrine growth factor) in the local breast tumor microenvironment makes the breast cancer cells rely heavily on factors involved in PRL signaling pathways, such as STAT3. The data presented in this study demonstrates that hPRL-G129R is able to specifically inhibit STAT3 phosphorylation in breast cancer cells. Although further clinical studies are needed to demonstrate the relevance of STAT3 activation and breast cancer, we believe that hPRL-G129R could potentially be a valuable addition to breast cancer therapy based on its abilities to inhibit PRL induced breast cancer cell proliferation, induce apoptosis and inhibit oncogene STAT3 phosphorylation.

Acknowledgments

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Combination of PCR Subtraction and cDNA Microarray for Differential Gene Expression Profiling

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ABSTRACT

PCR subtraction hybridization has been used effectively to enrich and single out differentially expressed genes. However, identification of these genes by means of cloning and sequencing individual cDNAs is a tedious and lengthy process. In this report, an attempt has been made to combine the use of PCR select cDNA subtraction hybridization and cDNA microarrays to identify differentially expressed genes using a nonradioactive chemiluminescent detection method. mRNA from human prolactin (hPRL) or human prolactin antagonist (hPRL-G129R) treated and non-treated breast cancer cells was isolated, and cDNAs were synthesized and used for the PCR subtraction to enrich the differentially expressed genes in the treated cells. The PCR-amplified and subtracted cDNA pools were purified and labeled using the digoxigenin method. Labeled cDNAs were hybridized to a human apoptosis cDNA microarray membrane and identified by chemiluminescence. The results suggest that the strategy of combining all three methods will allow for a more efficient, nonradioactive way of identifying differentially expressed genes in target cells.

INTRODUCTION

Our previous studies have shown that hPRL demonstrates a stimulatory effect on human breast cancer cell proliferation (1). We have also reported that an hPRL mutant with a single amino acid substitution mutation at position 129 (hPRL-G129R) acts as an hPRL receptor antagonist on human breast cancer cells (1). Further investigation of hPRL-G129R demonstrated that its inhibitory effects on breast cancer cells are through the induction of apoptosis (1). However, the exact mechanism of hPRL-G129R-induced apoptosis is still awaiting further investigation.

To study the physiological mechanisms of different cell types and of cells under different conditions, PCR subtraction hybridization has been used widely over the years (4–6). This technique gives a representation of differentially expressed genes from one group of cells as compared with another. The theory behind the technique is very simple. It first uses mRNA from two populations of cells and converts them into cDNA. The cDNA from cells that contain differentially expressed genes is referred to as the "tester", and the reference cDNA is referred to as the "driver". Both tester and driver cDNAs are first digested using a 4 base-cutter restriction enzyme to create shorter blunt-ended molecules. The ends of the tester cDNAs are modified by ligating adaptors that will serve as PCR primers. The tester cDNAs are then hybridized with driver cDNAs, which have no adaptors on their ends. Suppression PCR, using the adaptors as primers, is then performed to allow exponential amplification of the differentially expressed genes.

Identifying these genes by the use of conventional methods such as cloning, sequencing, and northern blot analysis is a tedious and expensive process (6,9). Recently, the vast emergence of the cDNA microarray techniques greatly expands the ability of researchers to identify previously cloned sequences in pools of cDNAs. This technique has proved to be an essential tool when trying to identify which genes are responding to a certain condition (6,9). One of the drawbacks of using directly isolated mRNA/cDNA as probes in screening commercial membranes is a high signal-to-noise ratio (2). In this

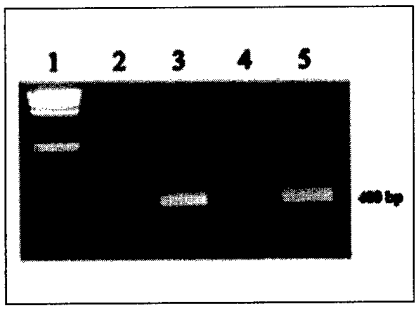

Figure 1. Efficiency test of the PCR select subtraction hybridization. The housekeeping gene G3PDH (approximately 400-bp fragment) amplified for 12 cycles with primers provided from PCR Select cDNA Subtraction Hybridization kit on a 1% agarose/ethidium bromide gel. Lane 1 is λ DNA/*Hin*dIII-digested molecular weight marker. Lanes 2 and 4 are subtracted Testers A and B, respectively, and lanes 3 and 5 are the unsubtracted tester controls.

study, we combine the use of suppression subtractive hybridization to enrich differentially expressed cDNAs upon treatment with hPRL and hPRL-G129R. We will then identify the differentially expressed cDNAs by the use of microarray technology. Also, nucleotide hybridization detection without the use of radioisotopes is of interest to many researchers (3,7,10,11). Therefore, we employed the use of a nonradioactive chemiluminescent system, digoxigenin (DIG), to label the cDNAs as an alternative way of identifying genes on the microarray membrane.

MATERIALS AND METHODS

Cell Lines and Growth Conditions

The T-47D human breast cancer cell line was obtained from ATCC (Manassas, VA, USA). Cells were maintained in phenol red-free RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) (both from Invitrogen, Carlsbad, CA, USA) and grown at 37°C in an atmosphere containing 5% CO_2. Before treatment with hPRL or hPRL-G129R, cells were split into three groups (10 T75 flasks were used for each group) and depleted with RPMI 1640 supplemented with 10% Charcoal/Dextran-treated FBS (CSS) for six days until cells reached 80% confluency. After depletion, approximately 10^8 cells from each group were treated with either 500 ng/mL hPRL (Tester A) (hPRL was kindly supplied by Dr. A.F. Parlow, National Hormone and Pituitary Program, NIH, USA) in RPMI 1640 supplemented with 1% CSS or 500 ng/mL of hPRL-G129R (Tester B) (hPRL-G129R was produced in our laboratory; Reference 8) or cultured with 1% CSS alone as the untreated control (Driver). Cells were treated for 48 h, and then mRNA was isolated in the following section.

PCR cDNA Subtraction Hybridization

mRNA isolation was performed using the Micro-Fast Track™ 2.0 kit from Invitrogen according to the manufacturer's instructions. RNA yield was determined by measuring absorbance at 260 nm. Subtractive hybridization was performed using the PCR Select™ cDNA Subtraction Kit from Clontech Laboratories (Palo Alto, CA, USA). Briefly, 10 µg mRNA were used for synthesizing Tester A, Tester B, and Driver cDNAs. Restriction enzyme digests of the cDNAs, adaptor ligation of the testers, and two rounds of hybridization between tester and driver were carried out following the manufacturer's instructions. After the hybridizations were complete, primary PCR was used to amplify the products. Conformation of the subtractions was performed on the primary PCR products using the primers for the housekeeping gene, G3PDH, supplied in the kit.

Generation of enriched cDNAs, which will be labeled and used as probes for screening the microarray, were prepared with a secondary PCR. Four 50-µL reactions were performed and pooled. cDNAs were purified using QIAquick™ PCR Purification Kit (Qiagen, Valencia, CA, USA). DNA yield was determined by measuring the absorbance at 260 nm.

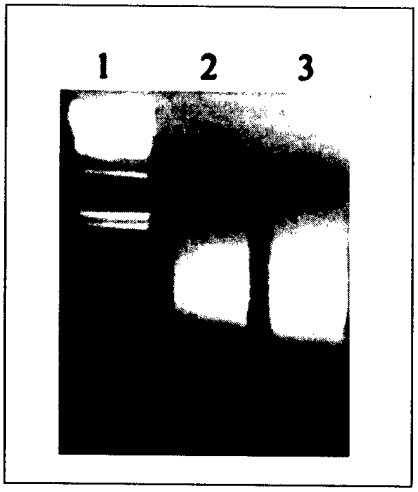

Figure 2. Analysis of enriched cDNAs from PCR select subtraction hybridization. After the secondary PCR, a sample of the purified hPRL-specific cDNAs (lane 2) and hPRL-G129R-specific cDNAs (lane 3) was run on a 1% agarose/ethidium bromide gel. Lane 1 is λ DNA/*Hin*dIII-digested molecular weight marker.

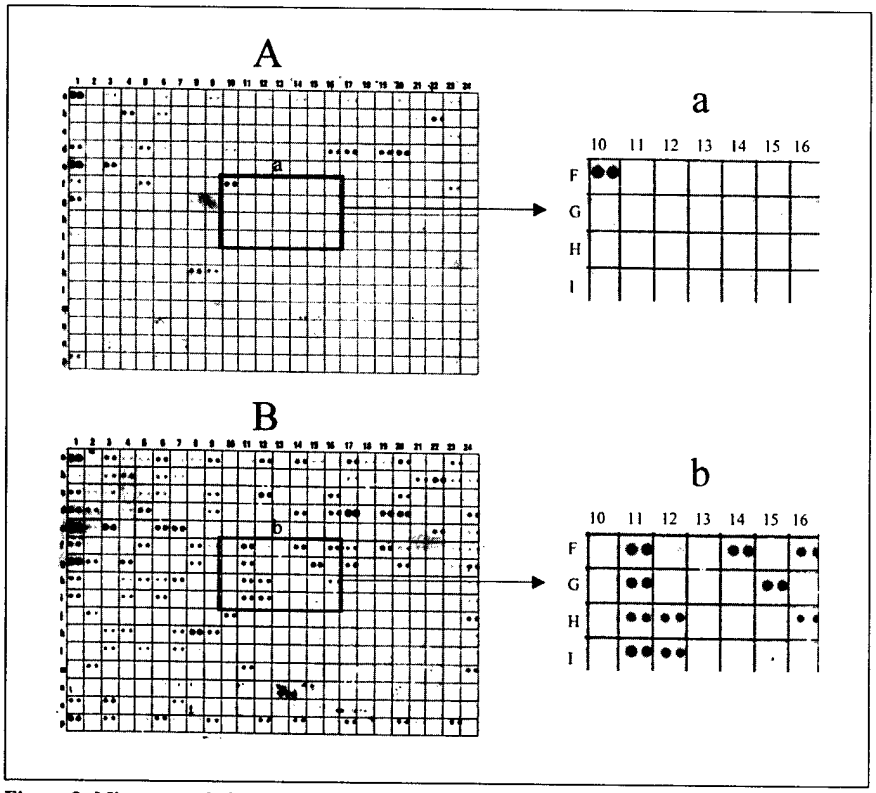

Figure 3. Microarray hybridized with enriched subtracted cDNAs. Human apoptosis cDNA microarrays were hybridized with DIG-labeled, hPRL-specific cDNAs (A) or hPRL-G129R-specific cDNAs (B). A section of the hPRL-specific array (a) or hPRL-G129R-specific array (b) was enlarged for comparison purposes. We have identified that cDNAs represented by 10F in the hPRL-treated cancer cells and various others in the hPRL-G129R-treated cells are of special interest.

Labeling of cDNA Probes

Purified cDNAs from the PCR subtraction hybridization were randomly primed and labeled with DIG-dUTP, alkali-labile DNA Labeling Kit (Roche Molecular Biochemicals, Mannheim, Germany). Three micrograms of cDNA were labeled according to the manufacturer's protocol and incubated for more than 20 h. An overnight incubation was performed, as previous results have demonstrated, to ensure an efficient yield of newly synthesized DIG-labeled DNA.

cDNA Microarray

Atlas™ Human Apoptosis Arrays from Clontech Laboratories containing all currently known apoptosis related genes on a nylon membrane were prehybridized with DIG Easy Hyb® solution (Roche Molecular Biochemicals) at 37°C for 2 h in a hybridization incubator with gentle rotation. Three micrograms of DIG-labeled probes were purified and resuspended in 20 µL distilled water. The probes were boiled for 10 min and quickly chilled on ice for ≥ 5 min. After pre-hybridization, 20 µL DIG-labeled probe were added to the microarray membrane in which 5 mL fresh DIG Easy Hyb had been added. Membranes were hybridized overnight at 68°C in a hybridization incubator with gentle rotation. The following day, membranes were washed at 38°C twice (5 min/wash) in 2× standard saline citrate (SSC), 1% SDS, and twice at 68°C (15 min/wash) in 0.1× SSC, 0.5% SDS.

Chemiluminescent Detection

Hybridized Atlas membranes were developed using the DIG Luminescent Detection Kit (Roche Molecular Biochemicals) according to the manufacturer's specifications. CSPD® (Roche Molecular Biochemicals) was used as the chemiluminescent substrate. After 5 min of incubation with CSPD, membranes were wrapped in plastic wrap, placed in an autoradiography cassette, and incubated at 37°C for 15 min. The membrane was then exposed to Kodak® Biomax™-MR film (Eastman Kodak, Rochester, NY, USA) at room temperature for various amounts of time to obtain an optimal exposure.

RESULTS AND DISCUSSION

In this report, we successfully combined three proven effective commercially available methods to profile genes in breast cancer cells in response to various treatments using nonradioactive techniques.

When performing the mRNA isolation, we have found that it is important to start with at least 10^8 cells to obtain sufficient mRNA for completing the experiment. We have found that a minimum of 10 µg mRNA should be used (instead of 2 µg as recommended) to produce the optimal amount of cDNAs for the remainder of the experiment. Once the primary and secondary PCRs are completed, an efficiency test must be performed to verify the subtraction efficiency. As shown in Figure 1, after two separate PCR runs were performed, the G3PDH was greatly reduced in the subtracted samples as compared to the unsubtracted.

To obtain enough cDNAs to be used for labeling and probing of the microarray, we recommend pooling multiple secondary PCRs together when making the cDNA probes. Figure 2 shows the results of the cDNA enrichment reactions. The cDNA shown on the gel represents 1/10 of the purified pool of cDNAs obtained from the final PCR product (keep in mind that housekeeping genes such as G3PDH could not be amplified after subtraction; see Figure 1). A total of 3 µg amplified final PCR-produced cDNAs was used for labeling with DIG to increase the amount of genes to be labeled properly.

To search for any apoptosis-related genes present in the cDNA pools after hPRL or hPRL-G129R treatments, an Atlas human apoptosis cDNA microarray containing 205 apoptosis related genes was used. Figure 3 shows the results after hybridization with the DIG-labeled cDNAs. There are distinct differences found with the treatment of either hPRL or hPRL-G129R. Many apoptosis-related genes are being expressed in the hPRL-G129R-treated cells, as would be expected since previous experiments showed it to induce apoptosis. Various caspases are shown in Figure 3, lane 11. There is no presence of these genes in the hPRL-treated cDNAs, as shown in Figure 3.

These results prove that, by combining the use of PCR select cDNA subtraction hybridization, cDNA microarrays, and chemiluminescent detection, one is able to detect and identify differentially expressed genes. This technique is valuable in that it can be performed with any desired cell lines, with any treatment of the researcher's choice, and probed using any variation of cDNA microarray membranes. These methods together will allow researchers the ability to target and study specific differentially expressed genes in a cost-efficient and environmentally friendly manner.

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Quantification of prolactin receptor mRNA in multiple human tissues and cancer cell lines by real time RT-PCR

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Abstract

Human prolactin (hPRL) has been reported to be involved in breast and prostate cancer development. The hPRL receptor (hPRLR) is expressed in a wide variety of tissues in at least three isoforms. In this study, a one-step real time reverse transcription PCR technique was used to determine relative expression levels of hPRLR mRNA in eleven human breast cancer cell lines, HeLa cells, three prostate cancer cell lines and nine normal human tissues. The housekeeping gene β-actin was used for internal normalization. We demonstrate that hPRLR mRNA is up-regulated in six of the eleven breast cancer cell lines tested when compared with normal breast tissue. Of the cancer cell lines tested, we found that T-47D cells have the highest level of hPRLR mRNA, followed by MDA-MB-134, BT-483, BT-474, MCF-7 and MDA-MB-453 cells. In two breast cancer cell lines (MDA-MB-468 and BT-549), the hPRLR levels were found to be comparable to that of normal breast tissue. Three breast cancer cell lines (MDA-MB-436, MDA-MB-157 and MDA-MB-231) expressed hPRLR mRNA at levels lower than that of normal tissue. In contrast, in all three commonly used prostate cancer cell lines (LNCaP, PC-3 and DU 145), the levels of hPRLR mRNA were found to be down-regulated relative to that of normal prostate tissue. Of nine normal human tissues tested, we found that the uterus and the breast have the highest levels of hPRLR mRNA, followed by the kidney, the liver, the prostate and the ovary. The levels of hPRLR mRNA were the lowest among the trachea, the brain and the lung.

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Introduction

The prolactin receptor (PRLR) belongs to the cytokine receptor superfamily. PRLR consists of three domains: the extracellular ligand binding domain, the transmembrane domain and the proline-rich cytoplasmic domain. Following PRL and PRLR interaction, signal transducers and activators of transcription (STATs) are ultimately phosphorylated prior to binding to PRL-responsive promoter elements in the nucleus resulting in PRL action (Bole-Feysot *et al.* 1998; Clevenger & Plank 1997; Das & Vonderhaar 1997). The evidence linking PRL to breast cancer development has been drawn, in part, from findings of higher PRLR levels in cancerous tissues (Laud *et al.* 2000, Ormandy *et al.* 1997, Reynolds *et al.* 1997, Touraine *et al.* 1998). Experimentally, over-expression of PRL in mice results in a high incidence of mammary tumors. In humans, there is a positive correlation between PRLR, estrogen receptor (ER) and progesterone receptor levels, and it is known that sex steroid hormones and PRL interact synergistically to initiate cancerous growth within mammary tissue (Ormandy *et al.* 1997). There is growing evidence that PRL may also play a role in early transformation events involved in prostate cancer (Costello *et al.* 1999), and that PRLR expression is altered in some neoplasms of the prostate (Leav *et al.* 1999). More importantly, the PRL antagonist hPRL-G129R, which blocks PRLR signal transduction, appears to induce breast cancer cell apoptosis (Cataldo *et al.* 2000). Therefore, we found it of interest to quantitate PRLR mRNA levels of breast and prostate human cancer cell lines and compare these directly to normal tissue levels. Ultimately, this information will be useful in the selection of cell lines for PRL-related studies based on PRLR status.

Materials and Methods

Cell lines and tissues

The following human cancer cell lines were obtained from the ATCC and maintained under the conditions recommended. We collected eleven human breast cancer cell lines (MCF-7, T-47D, MDA-MB-134, BT-483, BT-474, MDA-MB-453, MDA-MB-468, BT-549, MDA-MB-436, MDA-MB-157, and MDA-MB-231); three prostate cancer cell lines (LNCaP, PC-3, and DU 145);

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and the HeLa cell line. Seven tissue total RNA preparations were obtained from Clontech Lab, Inc. (adult brain, kidney, liver, lung, trachea, uterus and prostate), and two from Stratagene, Inc. (adult breast and ovary).

Real-time quantitative PCR

A one-step real time reverse transcription (RT) PCR technique was used to determine relative expression levels of PRLR mRNA using the ABI Perkin Elmer Prism 7700 Sequence Detection System (Applied Biosystems). For analyses from cell cultures, total RNA was isolated from 70–90% confluent cell cultures, using the RNAqueous (Ambion) RNA isolation kit following the recommended protocol. The reaction mix included a 200 nm final concentration of both forward (derived from exon 7: 5′agaccatggatactggagta-3′) and reverse (derived from exon 9: 5′ggaaagatgcaggtcaccat-3′) PRLR-specific primers, and a 100 nm final concentration of the PRLR specific probe (5′tctgctgtcatctgtttgatta-3′) labeled with FAM reporter fluorescent dye; these primers were designed for amplification of all three isoforms of PRLR. A one-step reaction mixture provided in the TaqMan Gold RT-PCR Kit (PE Applied Biosystems) was used for all amplifications (5·5 mM MgCl2, 50 mM KCl, 0·01 mM EDTA, 10 mM Tris–HCl pH 8·3, 300 μM deoxyATP, 300 μM deoxyCTP, 300 μM deoxyGTP, 600 μM deoxyUTP, 0·025 U/ml AmpliTaq Gold DNA polymerase, 0·25U/ml MultiScribe Reverse Transcriptase, 0·4 U/ml RNase inhibitor).

Cycle parameters for the one-step RT-PCR included a reverse transcription step at 48 °C for thirty min, followed by 40 cycles of 95 °C denaturation and 60 °C annealing/extension. Four hundred to 1500 nanograms of total RNA were used per reaction; the housekeeping gene β-actin was used for internal normalization. For analyses of PRLR in normal tissues, 100 nanograms of commercially-prepared total RNA were used per reaction. Each reaction was carried out in triplicate and repeated at least three times. Data were expressed as the means ± S.E.

Results and Discussion

Our results from RT-PCR demonstrate that T-47D cells express the highest levels of PRLR mRNA (Figures 1 and 2) among the cell lines tested. The levels of PRLR mRNA in breast cancer cell lines are much higher than those of prostate cancer cells (Fig. 3). PRLR mRNA was not detectable in HeLa cell RNA preparations (Fig. 3).

Within the panel of normal tissues, uterus and breast expressed the highest levels of PRLR mRNA (Fig. 4). We set the expression level from breast tissue to 1, to allow internal comparisons between tissues. We found that PRLR mRNA expression from the kidney was surprisingly high, suggesting an important role for PRL in this tissue.

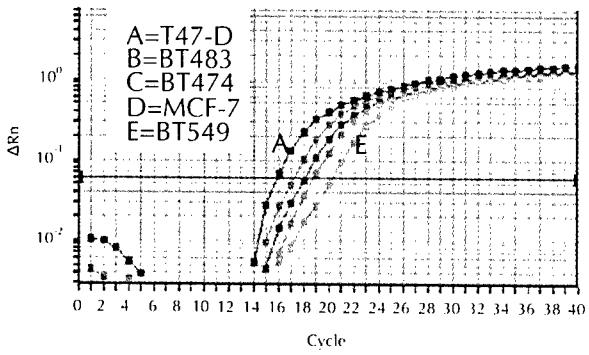

Figure 1 Real-time RT-PCR analysis for PRLR mRNA in five breast cancer cell lines (A-E, from left to right as indicated).

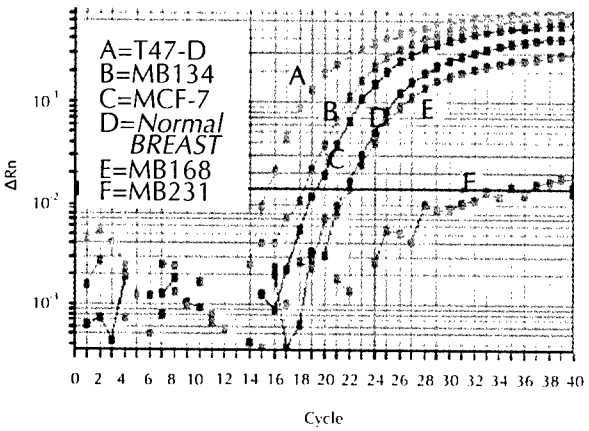

Figure 2 Real-time RT-PCR analysis for PRLR mRNA in breast cancer cell lines (A-C), normal breast tissue (D) and MDA-MB-468 (E), and MDA-MB-231 (F).

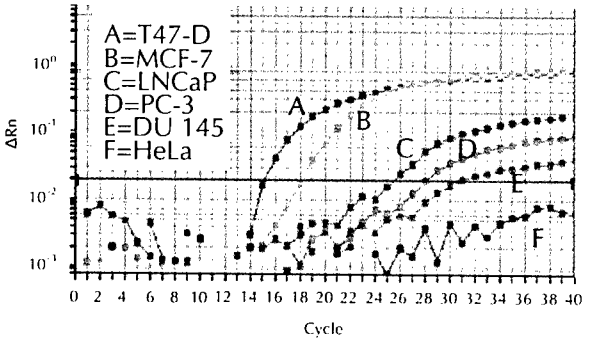

Figure 3 Real-time RT-PCR analysis for PRLR mRNA in breast cancer cell lines (A, B), prostate cancer cell lines (C-E), and the HeLa cell line (F).

In order to directly compare the expression levels of PRLR between the cell lines and tissue preparations, RT-PCR reactions were carried out using 100 ng of total RNA of normal mammary or prostate tissues and 100 ng of total RNA from several breast or prostate cancer cell lines. We found that the PRLR mRNA expression level of normal breast tissue was comparable to that of the cell

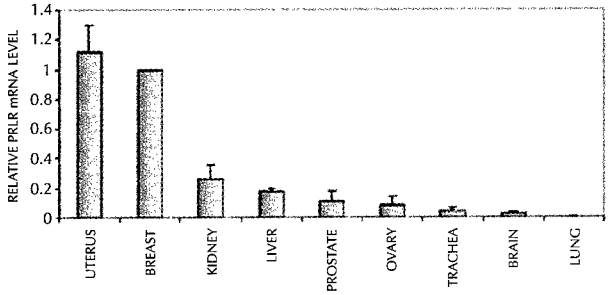

Figure 4 Comparison of relative PRLR mRNA levels in nine normal human tissues. Y-axis, fold difference.

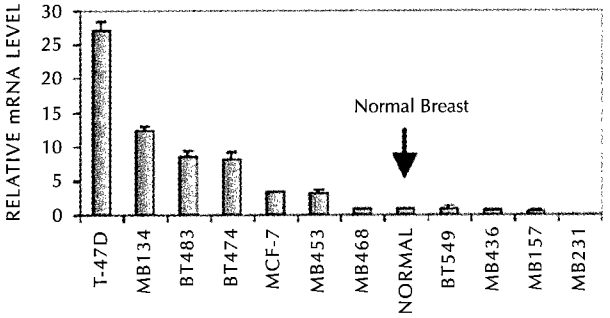

Figure 5 Comparison of relative PRLR mRNA level in eleven breast cancer cell lines and normal breast tissue, normalized to 100 ng of total RNA. Y-axis, fold difference.

line MDA-MB-468 (Fig. 2). Therefore, PRLR mRNA expression levels in MDA-MB-468 cells were used to normalize relative expression level from all cell lines by adjusting all β-actin values to β-actin amplification levels from one ug of MDA-MB-468 total RNA. A graphical representation and summary table of these findings are presented in Figure 5 and Table 1.

Table 1 Relative hPRLR mRNA levels in human cancer cell lines

	Fold difference (± S.E.)
Cell lines	
T-47D	27·20 (1·24)
MDA-MB-134	12·45 (0·55)
BT483	8·62 (0·76)
BT474	8·13 (1·03)
MCF-7	3·45 (0·06)
MDA-MB-453	3·17 (0·55)
MDA-MB-468	**1·0***
BT549	**1·0 (0·28)**
MDA-MB-436	0·69 (0·07)*
MDA-MB-157	0·62 (0·07)
LNCaP	0·006 (0·0005)
PC-3	0·002 (0·0002)
MDA-MB-231	0·0017 (0·0064)
DU145	0·00032 (0·00001)
HeLa	not detected

*Not detected by Northern blotting methods.

Table 2 Relative hPRLR mRNA levels: comparison between two studies

	Current study	**Ormandy *et al.* (1997)**
Cell lines		
T-47D	7·9	4·0
MDA-MB-134	3·6	5·2
BT483	2·5	4·0
BT474	2·4	2·3
MCF-7	**1·0**	**1·0**
MDA-MB-453	0·9	0·7
MDA-MB-468	0·3	ND*
BT549	0·3	0·6
MDA-MB-436	0·2	ND*
MDA-MB-157	0·2	0·4
MDA-MB-231	0·0005	ND*

*Not detected by Northern blotting methods.

The results using this method were compared with those published earlier in which Northern blotting methods were used to determine relative hPRLR mRNA levels in human breast cancer cell lines (Table 2). Although the two methods generated similar values in most cases, we were able to detect PRLR mRNA expression in cell lines that had previously been noted to lack PRLR expression (MDA-MB-468 and MDA-MB-436). The finding of higher expression levels of PRLR mRNA in four cell lines (T-47D, MDA-MB-134, BT-483 and BT-474) is consistent with the findings of Ormandy *et al.* (1997). Our findings are also consistent with those of Shiu *et al.* (1987), in which PRLR numbers were directly calculated in a relatively limited panel of breast cancer cell lines.

It should be noted that although the expression level of PRLR in the normal prostate tissue is moderately high, all three commonly used prostate cancer cell lines expressed extremely low but detectable levels of PRLR mRNA (Fig. 6), ranging from approximately 165 fold lower (LNCaP), and 460 fold lower (PC-3) to 3100 fold lower (DU 145) than MDA-MB-468 levels (Table 1). We are unsure if down-regulation of PRLR is a common phenomenon of prostate cancer. In any case, one should be aware of lower PRLR levels in these cell lines relative to normal prostate tissue (Fig. 6) when choosing these prostate cancer cell lines as study models.

Real-time quantitative PCR is a method proving to be invaluable in the analysis of a number of receptors involved in breast cancer and its metastasis, including prolactin and chemokine receptors (Muller *et al.* 2001). Although normal breast tissue expressed the second highest level of PRLR mRNA of the tissue samples, this level was less than a twentieth that of the malignant cancer cell line T-47D, and well below levels of five other mammary cancer cell lines, supporting a growing body of evidence that increased PRLR expression and prolactin activity

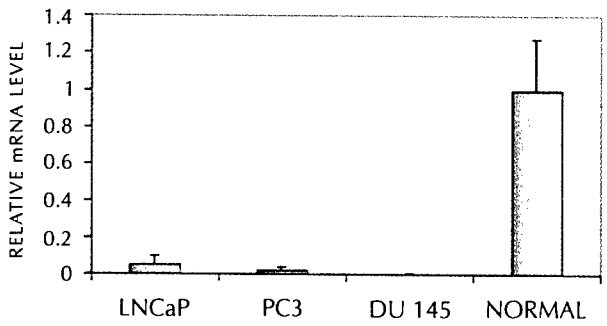

Figure 6 Comparison of relative PRLR mRNA levels of three prostate cancer cell lines and normal prostate tissue, normalized to 100 ng of total RNA. Y-axis, fold difference.

contribute to mammary carcinoma (Clevenger & Plank 1997; Ormandy *et al.* 1997; Reynolds *et al.* 1997; Touraine *et al.* 1998; Vonderhaar 1998; Vonderhaar 1999; Wennbo & Tornell 2000).

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In vivo studies of the anti-tumor effects of a human prolactin antagonist, hPRL-G129R

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Abstract. Previously we demonstrated that a mutated human prolactin (hPRL) with a single amino acid substitution at position 129 (hPRL-G129R) was able to inhibit human breast cancer cell proliferation via the induction of apoptosis. In this study, we report the *in vivo* anti-tumor effects of hPRL-G129R in nude mice bearing human breast cancer xenografts (T-47D and MCF-7). In an effort to prolong the half-life of the proteins, hPRL or hPRL-G129R were formulated with either growth factor reduced Matrigel or into slow-releasing pellets (custom made 5 mg/5 day release). Initially, nude mice inoculated (s.c.) with T-47D human breast cancer cells were treated with either hPRL or hPRL-G129R formulated with Matrigel. At the end of the 7-week study, it was found that hPRL significantly stimulated the *in vivo* growth of T-47D xenografts (mean tumor volume, 202±62 mm^3 as compared to 124±31 mm^3 in control mice), whereas hPRL-G129R inhibited the tumor growth (mean tumor volume, 79±32 mm^3). The inhibitory effects of hPRL-G129R were further confirmed in a second experiment using nude mice bearing MCF-7 human breast cancer xenografts and treated with slow-releasing pellets containing hPRL-G129R. Based on these results, we believe that hPRL-G129R can be used to improve the outcome of human breast cancer treatment in the near future.

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Abbreviations: ER, estrogen receptor; E2, 17-ß estradiol; FBS, fetal bovine serum; FPLC, fast-performance liquid chromatography; hPRL, human prolactin; hGH, human growth hormone; IRMA, immunoradiometric assay; STAT, signal transducers and activators of transcription

Key words: prolactin antagonist, breast cancer xenografts, nude mice

Introduction

Human PRL is a neuroendocrine polypeptide hormone primarily produced by the lactotrophs of the anterior pituitary gland in all vertebrates. The biological activities of PRL are mediated by a specific membrane receptor, the PRL receptor. Although hPRL has been reported to have multiple biological activities, the best-characterized action of PRL is on the mammary gland (1). In this organ, PRL plays a decisive role in DNA synthesis, epithelial cell proliferation and milk production (2-5). It has been unambiguously demonstrated in studies using PRL or PRL receptor gene knock-out mice that PRL and PRL receptors are the key regulators in mammary tissue development (5,6).

Recently, the notion that hPRL acts as a survival growth factor in the mammary gland and is directly involved in breast cancer development has revitalized the efforts in searching for a hPRL receptor blocker. In our previous studies (7-12), we developed an hGH antagonist with a single amino acid substitution mutation from Gly→Arg at position 120 (hGH-G120R). This peptide-based therapeutic has proven to be clinically effective in blocking the hGH receptor. Human GH antagonist has completed phase III studies and will be used in patients with pathological levels of GH (13). By adopting a strategy similar to the one used in the development of the hGH antagonist, we (14-16) and others (17-20) have demonstrated that a single amino acid substitution mutation (Gly→Arg at position 129) in the hPRL molecule results in an hPRL receptor specific antagonist (hPRL-G129R). We have demonstrated that hPRL-G129R is able to inhibit human breast cancer proliferation via the induction of apoptosis. We have also demonstrated that the possible mechanism of the hPRL antagonist's inhibitory effects is mediated, at least in part, through: a) inhibition of phosphorylation of oncogene STAT3 (15); b) modulation of TGFs (up regulation of TGFß and down regulation of TGFα) (16); and c) induction of caspase 3 activities (26). The purpose of this study is to further test the anti-tumor activities of hPRL-G129R *in vivo* using nude mice inoculated with human (T-47D or MCF-7; s.c.) mammary tumor cells and treated with hPRL-G129R using two different delivery methods intent to prolong the half-life of the proteins (the Matrigel mix and slow-releasing pellets). The results from four different *in vivo* experiments demonstrated that hPRL stimulates breast cancer cell growth and more importantly hPRL-G129R significantly inhibited breast cancer cell growth *in vivo*.

Materials and methods

Cell lines and animals. The cell lines used in this study were two human breast cancer cell lines (T-47D and MCF-7) from ATCC (Manassas, VA). T-47D cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (Gibco BRL; Baltimore, MD) and ATCC recommended supplements. MCF-7 cells were grown in DMEM supplemented with 10% FBS. Six- to 8-week-old female Nuj/nude mice were obtained from the Jackson Lab (Bar Harbor, ME) and maintained in a sterile environment in compliance with NIH guidelines. Animals were allowed to adjust to the institutional animal facility for 1 week before the experiment.

Production and purification of hPRL and hPRL-G129R. Human PRL and hPRL-G129R used in this study were produced using an *E.coli* protein production system according to published protocols (15,20) with modifications. Briefly, BL21 (DE3) cells (Novagen; Madison, WI) were transformed with hPRL or hPRL-G129R expression plasmids (pET22b-hPRL or pET22b-G129R) using the calcium chloride method. The transformant was spread on an ampicillin plate, and grown overnight at 37°C. An LB seed culture was inoculated with 6-10 colonies and incubated overnight. The following day, an LB culture was generated by inoculation of 5% of the seed culture and grown for ~2.5 h at 37°C with agitation. IPTG (Fisher Scientific; Norcross, GA) was then added to the culture (1 mM final concentration) to induce expression of hPRL or hPRL-G129R and incubated for an additional 4 h. Bacteria were pelleted and resuspended in a solution containing 0.2 M $NaPO_4$ (pH 8.0), 10 mM EDTA, and 0.5% Triton X-100. The resuspended cells were lysed using a 550 Sonic Dismembrator from Fisher Scientific (Norcross, GA), and the products in the form of inclusion bodies were pelleted by centrifugation at 12,000 g for 15 min. The pellets were then resuspended in solution A [0.2 M $NaPO_4$ (pH 7.0), 5 mM EDTA, 1 M urea, 0.5% Triton X-100] and pelleted by centrifugation at 12,000 g for 15 min. These pellets were then resuspended in solution B [0.2 M $NaPO_4$ (pH 8.0), 8 M urea, 1% v/v ß-mercaptoethanol], and the refolding process was initiated. The refolding process consisted of dialyzing the protein against decreasing amounts of urea and ß-mercaptoethanol in the presence of 50 mM NH_4HCO_3 (pH 8.0) for at least 3 consecutive days. The protein product was then filtered through a 0.22 µ filter, degassed and purified using a Q-Sepharose anionic exchange column (Pharmacia; Piscataway, NJ) on the FPLC system (Pharmacia; Piscataway, NJ). The concentration of hPRL or hPRL-G129R purified from FPLC was determined using the PRL immunoradiometric assay (IRMA) kit (DPC; Los Angeles, CA). The purity of both PRL and hPRL-G129R exceeded 98% as determined by SDS-PAGE in combination with silver staining (Bio-Rad; Hercules, CA). The endotoxin level in the final products from all batches was <5 EU/mg tested by Cape Cop Inc. The recombinant proteins produced by this method has an extra Met at the N-terminus as compared to wild-type PRL. The biological function of hPRL and hPRL-G129R was confirmed by the STAT assay as described previously (15).

Radioreceptor binding assay. Human PRL receptor binding assays were performed as previously described (14). Briefly, cells were grown in 6-well tissue culture plates until 90% confluent (~$1x10^5$ cells/well). Monolayers of cells were starved in serum-free RPMI-1640 medium for 0.5-1 h. The cells were then incubated at room temperature in serum-free RPMI-1640 containing $5x10^4$ cpm ^{125}I hPRL (specific activity, 30 µCi/µg; Perkin Elmer Life Sciences; Boston, MA) with or without 500 ng/ml of hPRL. Cells were washed three times in serum-free RPMI-1640, lysed with 0.5 ml of 0.1 N NaOH/1% SDS, and the bound radioactivity was determined by a scintillation counter. Total specific binding was calculated and compared.

Delivery of hPRL and hPRL-G129R. The *in vivo* half-lives of hPRL and hPRL-G129R are less than 2 h due to their small molecular sizes. Therefore, two alternative protein delivery methods were used in this study to extend the half-lives of these proteins. Serum PRL or PRL-G129R level was determined by PRL immunoradiometric assay (IRMA) kit.

Formulation with growth factor reduced Matrigel. Matrigel (BD Biosciences; San Diego, CA) is an artificial extracellular matrix that exists in liquid form at 4°C and solidifies into a gel at room temperature. Lyophilized hPRL or hPRL-G129R proteins were first hydrated with PBS (pH 8.0) and were then mixed with growth factor reduced Matrigel at a 1:1 or 1:2 (Protein:Matrigel, v/v) ratio to a final concentration of 1 mg/ml before injection. The *in vivo* phamocokinetics of protein/PBS and protein/PBS/Matrigel formulations were compared.

Formulation with slow-releasing pellets. Purified hPRL-G129R protein was lyophilized and sent to Innovative Research of America, Inc. (Sarasota, FL) for production of slow-releasing pellets. The pellets were implanted s.c. into experimental animals. Preliminary experiments demonstrated that 5 mg/5 day slow-releasing pellets resulted in satisfactory serum concentrations and minimal wounding. This formula was employed for the remainder of the study.

Tissue distribution of hPRL-G129R. We investigated the tissue distribution pattern of hPRL-G129R after i.p. injection into nude mice. FPLC purified hPRL-G129R was iodinated using ^{125}I (Perkin Elmer Life Sciences; Boston, MA). Approximately 0.5 µCi of ^{125}I labeled hPRL-G129R was i.p. injected into each of 10 nude mice bearing either T-47D (n=6) or MCF-7 (n=4) xenografts (7 weeks after initial tumor cell inoculation). Six hours after injection, animals were sacrificed and tissues were dissected and weighed. The radioactivity in the various tissues was determined using a scintillation counter. The data were expressed as cpm/mg tissue and normalized by reference to serum cpm (% tissue CPM = cpm in tissue/mg/cpm of 100 µl serum x 100).

In vivo inhibition of tumor growth studies

Experiment one. Five million T-47D cells pre-mixed with the Matrigel were injected into the mammary fat pads of 30 Nuj/nude mice, which were then implanted s.c. with slow-releasing E2 (17-ß estradiol) pellets (0.72 mg/60 day, Innovative Research of America, Inc.) to enhance tumor growth. Three days after tumor cell inoculation, the mice were randomized into three groups. Each animal was then injected s.c. five times a week with either Matrigel alone (150 µl/mouse) or

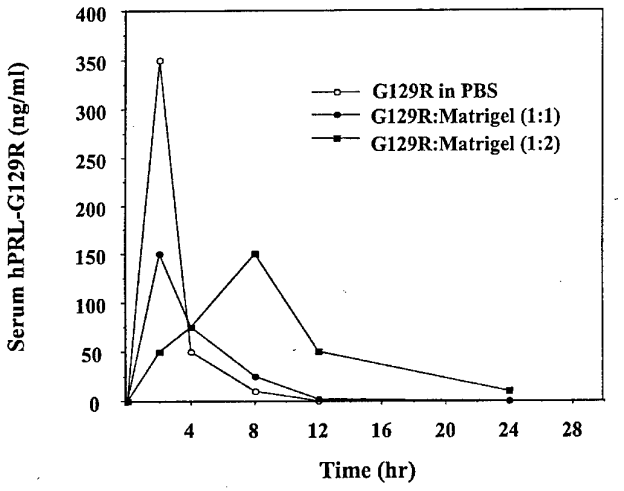


Figure 1. Pharmacokinetic studies of hPRL-G129R formulated with growth factor reduced Matrigel in mice. One hundred and fifty µg of hPRL-G129R were mixed with either Matrigel at a 1:1 or 1:2 v/v ratio (hPRL-G129R: Matrigel) or in PBS in a total volume of 150 µl and i.p. injected at time 0. Blood samples were collected at time intervals as indicated via tail vein bleeding. Serum hPRL-G129R levels were tested using an hPRL IRMA kit. Each data point represents the mean value from three animals.

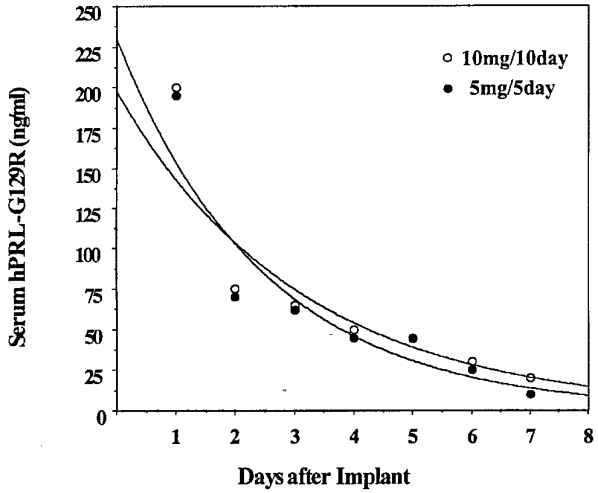


Figure 2. Pharmacokinetic studies of hPRL-G129R slow-releasing pellets. One hPRL-G129R slow-releasing pellet (5 mg/5 day or 10 mg/10 day) was s.c. implanted into each Balb/C mouse (n=3 for each group). Blood samples were collected daily via tail vein bleeding. The serum concentration of hPRL-G129R was tested using a PRL IRMA kit. Each data point represents the mean value from three animals.

Matrigel formulated (1:2 v:v ratio; 1 µg/ml) with hPRL-G129R or hPRL (150 µl/mouse) continuously for 7 weeks.

Experiment two. Since the original MCF-7 cells purchased from ATCC grow very slowly in nude mice (preliminary experiments, data not shown), secondary MCF-7 cultures were established. Briefly, a primary MCF-7 xenograft was established by injection of 10^7 MCF-7 cells into the mammary fat pad of a nude mouse in combination with an E2 slow-releasing pellet (s.c.). After the tumor was visible, it was dissected, minced and treated with trypsin. The tumor cells were then cultured and expanded. The sub-cultured MCF-7 cells were used to establish tumor xenografts in nude mice. Twelve Nuj/nude mice were inoculated with $5x10^6$ sub-cultured MCF-7 tumor cells, implanted s.c. with E2 pellets (0.72 mg/60 day) and then randomized into two groups. One group received implantation of slow-releasing hPRL-G129R pellets (5 mg/5 day), and the other group received implantation of placebo pellets. These mice received pellets once a week for 6 weeks.

Monitoring of tumor growth and statistics. Two dimensional tumor sizes were measured once a week. The tumor volume was calculated using the formula (L x W^2)/2. Tumors were dissected at the end of experiments and weighed. Assessment of statistical difference was determined by Student's t-test.

Results

Pharmacokinetics of hPRL-G129R formulated with the Matrigel or slow-releasing pellets. We compared the relative bio-availability and the duration of hPRL-G129R in serum using two different administration routes. The results demonstrated that both Matrigel formulations (1:1 or 1:2 ratio; v:v) of hPRL-G129R resulted in a more desirable serum profile than administration of hPRL-G129R/PBS. At the 1:2 ratio formulation, the peak concentration of hPRL-G129R was greatly reduced from approximately 350 ng/ml to approximately 150 ng/ml (Fig. 1). Also, the peak concentration in serum is delayed from 2 to 8 h, which resulted in much longer bio-available serum levels of hPRL-G129R (Fig. 1). Therefore, it is our belief that at a 1:2 (v:v) ratio mix, the protein:Matrigel formulation could be used as a novel protein delivery method. The 1:2 ratio is used throughout this study.

The second method of administration, implantation of slow-releasing pellets, resulted in an even more prolonged half-life of around 48 h (Fig. 2). In addition, significant serum hPRL-G129R concentrations continued to be detected 5 days after initial implantation. The average serum concentration was approximately 50 ng/ml 5 days after implantation. There was little difference in the serum profile between the two formulations (5 mg/5 day vs. 10 mg/10 day) for the slow-releasing pellets (Fig. 2). Considering the size of the pellets (directly related to the wound for each implantation), the 5 mg/5 day slow-releasing pellets were chosen for this study and were implanted once a week.

PRL receptor status in breast cancer cell lines. The results of a direct comparison of the PRL receptor specific binding levels in the three breast cancer cell lines used in this study are shown in Fig. 3. T-47D cells have the higher specific PRL receptor binding (55%), as compared to that of MCF-7 cells (25%). HeLa cells were included as a negative control. These results are consistent with findings from reverse transcriptase real time PCR assays conducted in our lab measuring expression of PRL receptor mRNA levels in these three cell lines (data not shown).

Tissue distribution. The distribution of ^{125}I hPRL-G129R 6 h after i.p. injection is represented in Fig. 4. The mammary

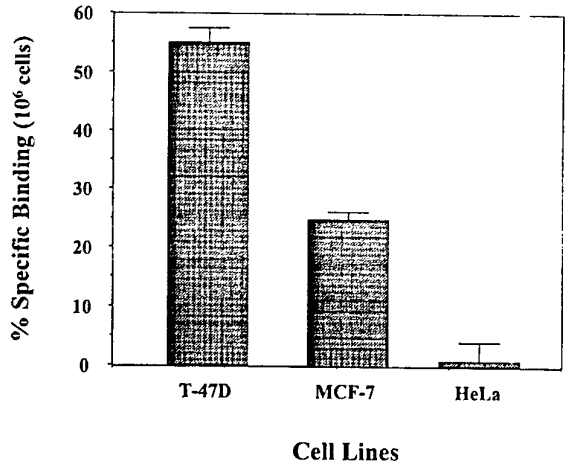


Figure 3. Radioreceptor binding assay was performed using ^{125}I labeled hPRL-G129R and three breast cancer cell lines. Specific binding of PRL receptor was measured using the formula: (cpm of total binding per 10^6 cells - cpm of non-specific binding)/cpm of total binding x 100, as described previously (14). A HeLa cell line was used as negative control.

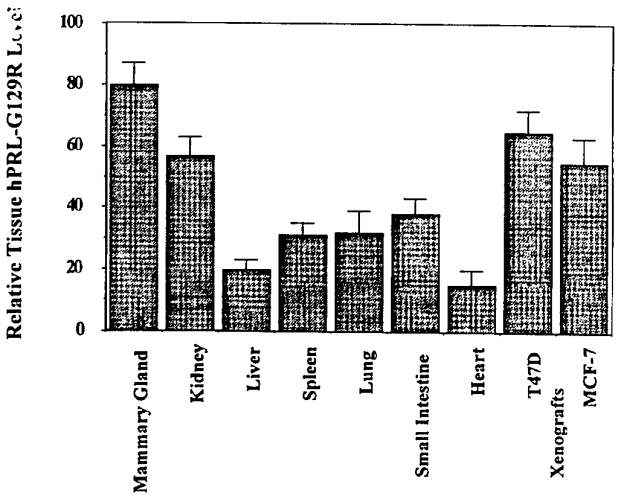
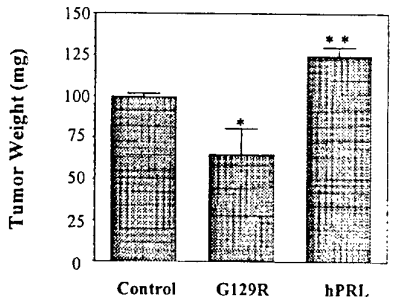


Figure 4. Tissue specific binding (pharmaco-distribution) of hPRL-G129R in nude mice bearing human breast cancer xenografts (T-47D, n=3; or MCF-7, n=3). One µCi of ^{125}I labeled hPRL-G129R was injected i.p. into experimental animals. Six hours after injection, animals were sacrificed and various tissues were dissected, weighed and the amount of radioactivity in each sample was determined by a scintillation counter. The data was normalized with reference to the cpm in 100 µl serum of each animal and expressed as relative cpm/mg tissue/cpm in 100 µl serum x 100.

gland and the breast cancer cell xenografts were the tissues containing the highest counts of ^{125}I hPRL-G129R. These findings are very important in supporting the use of an hPRL antagonist to target the mammary gland, especially the tumor cells. It is of interest that the counts in the kidney were much higher than other organs with rich blood supplies such as the liver and the lung, a finding that suggests the kidney might be one of the major organs involved in PRL metabolism. The levels of ^{125}I hPRL-G129R were lowest in the heart and the liver (Fig. 4).



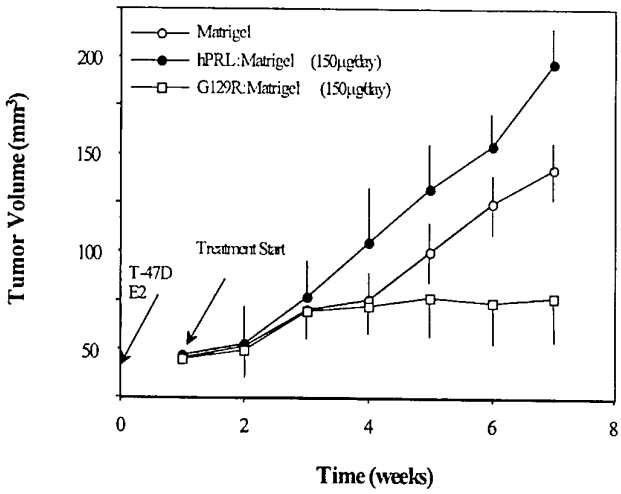


Figure 5. Effects of hPRL and hPRL-G129R on T-47D human breast cancer cell xenograft growth in nude mice. Thirty 6 to 7-week-old Nuj/nude mice were inoculated with T-47D cells and implanted s.c. with slow-releasing E2 pellets (0.72 mg/60 day). T47D cells ($5x10^6$) pre-mixed with Matrigel were injected into the mammary fat pad. One week after tumor cell inoculation, the mice were randomized into three groups and treated five times/week with either 150 µl of Matrigel (control), hPRL/Matrigel (150 µg/150 µl), or hPRL-G129R/Matrigel (150 µg/ 150 µl) for 7 consecutive weeks. The tumor volumes in each group were measured weekly. Tumors weights (mg) were taken at 7 weeks after tumor cell inoculation (upper panel). Values are expressed as mean and SE. *P<0.05; **P<0.01 vs. control.

In vivo inhibition of tumor growth

Experiment one: T-47D xenograft in nude mice treated with hPRL or hPRL-G129R/Matrigel mix. At the end of the 7-week period of treatment, Nuj/nude mice that had been implanted with T-47D cells and treated with the hPRL/Matrigel formulation exhibited enhanced tumor growth (mean tumor volume, 202±62 mm^3 vs. 124±31 mm^3 in the control mice). Those treated with the hPRL-G129R/Matrigel formulation showed inhibition of tumor growth (mean tumor volume was 79±32 mm^3 vs. 124±31 mm^3) (Fig. 5). While the tumor growth rate in the hPRL-G129R treated mice plateaued after the fifth week, tumor growth in the control and hPRL treated mice was clearly increasing beginning at around the fourth week of the experiment (Fig. 5). The final tumor weight in the three groups is also significantly different (P<0.05); (control, 100±2 mg; PRL, 121±5 mg; hPRL-G129R, 65±16 mg) (Fig. 5, upper panel).

Experiment two: MCF-7 xenograft in nude mice treated with hPRL-G129R slow-releasing pellets. Treatment with slow-

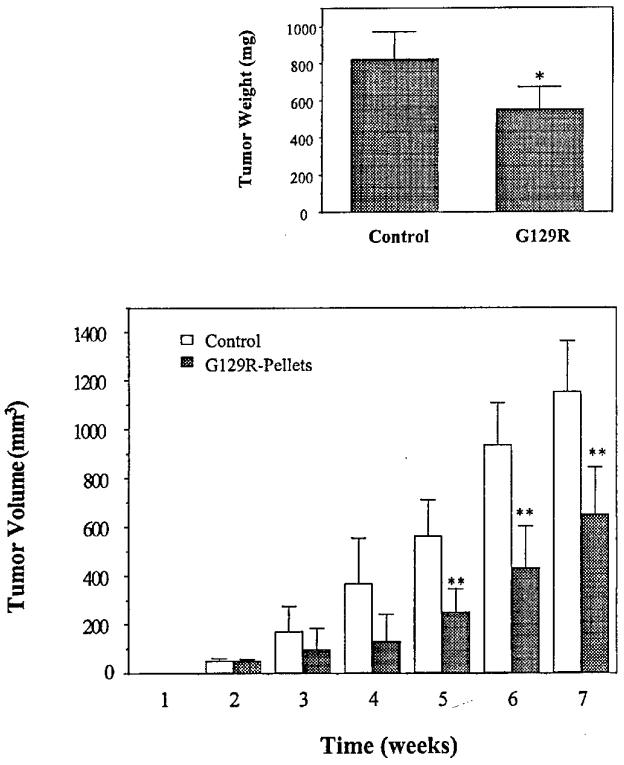


Figure 6. Effects of hPRL-G129R on MCF-7 human breast cancer cell xenograft growth in nude mice. Twelve 6- to 7-week-old Nuj/nude mice were inoculated with MCF-7 cells and implanted s.c. with slow-releasing E2 pellets (0.72 mg/60 day). MCF-7 cells ($5x10^6$) pre-mixed with Matrigel at 1:1 (v/v) ratio were injected into the mammary fat pad. Three days after tumor cell inoculation, the mice were randomized into two groups and received hPRL-G129R slow-releasing pellets or placebo (once/week) for 7 consecutive weeks. The tumor volumes in each group were measured weekly. Tumors weights (mg) were taken at 7 weeks after tumor cell inoculation (upper panel). Values are expressed as mean and SE. *P<0.05; **P<0.01 vs. control.

releasing hPRL-G129R pellets also resulted in inhibition of tumor growth in Nuj/nude mice inoculated with MCF-7 human breast cancer xenografts (Fig. 6). Since these secondary MCF-7 cells have been adapted to *in vivo* growth, they tend to grow much more aggressively as compared to original MCF-7 cells. At approximately 5 weeks after tumor inoculation, treatment with hPRL-G129R resulted in a decrease in tumor volume of about 50%. The tumor growth difference was most obvious between weeks 5 and 6. Along with tumor volume decreasing in hPRL-G129R treated nude mice, tumor weight also decreased as demonstrated in mice at 7 weeks of age bearing tumors (Fig. 6, upper panel).

Discussion

Estrogen is well known as a powerful mitogen that plays an important physiological role in human breast growth and function. The role of estrogen in breast cancer has also been well established and is supported by findings that anti-estrogen treatment has both therapeutic as well as preventive effects in the treatment of breast malignancies (21). However, the etiological role of hPRL as an autocrine/paracrine growth factor in breast cancer is still being challenged despite the fact that: a) hPRL has been shown to stimulate the proliferation of cultured breast cancer cells (14,22); b) high levels of hPRL receptor have been found in breast cancer tissues (23-25); and c) hPRL has been found to be produced locally in breast tissue (22). The controversy is largely due to the fact that there have been no convincing studies involving the use of anti-hPRL agents in an *in vivo* breast cancer model to establish the efficacy of an anti-PRL drug (26,27). In this report, we demonstrate that hPRL does indeed promote the growth of human breast cancer xenografts in nude mice (Fig. 5). More importantly, to the best of our knowledge, our data for the first time demonstrate the feasibility of using an hPRL antagonist to inhibit the growth of human breast cancer xenografts (Fig. 6).

The maintenance of relatively constant hPRL-G129R serum concentrations over a longer period of time is crucial for inducing the *in vivo* effects of the PRL antagonist. In this study, we used two alternative delivery methods to overcome the problem of a short hPRL-G129R half-life. As shown in Fig. 1, the peak serum concentration of hPRL-G129R was shifted from ~2 to 8 h after it is formulated with Matrigel, which resulted in a much longer serum half-life. Even more promising results were generated using the slow-releasing pellets of hPRL-G129R implanted once a week: in addition to a greatly extended half-life, hPRL-G129R serum concentrations were maintained within a range of 120 to 20 ng/ml for over a week (Fig. 2). We (14) and other groups (20) have, in the past, produced PRL antagonists that are highly effective in *in vitro* assays. This present study extends the therapeutic potential of hPRL-G129R protein.

The delivery methods used in this study are far from ideal from a clinical viewpoint. However, these two delivery methods provide alternatives to those used in peptide-based therapeutics. One obvious advantage of the delivery methods used in this study compared to those traditionally used to prolong the half-life of a protein (such as pegylation) is that they do not require chemical alteration of the therapeutic molecule. Therefore, functional testing is kept to a minimum before initiating *in vivo* studies.

Our tissue distribution studies provide some insight into the molecular nature of the therapeutic effects of hPRL-G129R treatment. Using ^{125}I labeled hPRL-G129R, it is clear that the human tumor xenografts contain high levels of hPRL-G129R-specific radioactivity, second only to mammary glands (Fig. 4). These findings indicate that high levels of the PRL receptor on the cancer cell surface provide the physical basis for the anti-tumor action of the PRL antagonist. We reason that the lower concentration of hPRL-G129R in tumor tissue vs. that of the mammary gland is due to the fact that the weight of the solid tumor masses dissected in these studies were as high as 900 mg (Fig. 6, upper panel); blood circulation in these solid tumors is much reduced relative to normal mammary tissue. We also note the higher levels of radioactivity in the kidney, higher even than that of the liver (Fig. 4), suggesting that the kidney may have high level of PRL receptors and is a target tissue of PRL as an osmoregulator.

The results from this study provide strong evidence suggesting that hPRL is a survival/growth factor for human breast cancer cells. By blocking the hPRL receptor with the mutated hPRL molecule (hPRL-G129R), we believe the

proliferative signaling pathways in the breast cancer cells are reversed. The exact molecular mechanism involved in this process is awaiting further elucidation. However, in our recent studies we have successfully used combination techniques of PCR-Select cDNA subtraction hybridization and cDNA microarrays to study the possible molecular mechanisms involved in the regulation of mammary gland apoptosis by hPRL (Beck MT, *et al*, 83th Annual Meeting of Endocrine Society, p199, 2001). Our preliminary results from hPRL treated T-47D cells revealed that out of the 205 apoptosis related genes only 1 gene, bcl-2, was up regulated in response to hPRL (bcl-2 is known as an apoptosis suppressor). On the other hand, many apoptosis related genes, in particular various caspases (3 and 7), Fas-activated serine/threonine (FAST) kinase, members of the tumor necrosis factor (TNF) family, and E2F were up regulated in hPRL-G129R treated T-47D cells (28). These results suggest that hPRL serves as an apoptosis inhibitor possibly through activation of bcl-2. Further studies are needed to confirm this observation.

In summary, we have successfully demonstrated that two protein delivery methods used in this study are able to maintain relatively stable concentrations of the hPRL-G129R in serum. Our results also indicate that hPRL contributes significantly to the growth of breast cancer *in vivo*. More importantly hPRL-G129R, the hPRL antagonist, was proven to be functionally active and successfully inhibited the growth of human breast cancer xenografts in nude mice. Together these results strongly indicate that the development of hPRL receptor antagonists will contribute significantly to the treatment of breast cancer.

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In vitro studies of a prolactin antagonist, hPRL-G129R in human breast cancer cells

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Abstract. Human prolactin (hPRL) has been shown to be one of the important survival/growth factors that promotes the proliferation of breast cancer cells in an autocrine/paracrine manner. In our recent studies, we demonstrated that a hPRL antagonist with a single amino acid substitution mutation (hPRL-G129R) was able to inhibit breast cancer cell proliferation via induction of apoptosis (1). In this study three independent yet related experiments were carried out regarding the effects of hPRL-G129R in breast cancer cells. We investigated the possible mechanism(s) of hPRL-G129R induced apoptosis in breast cancer cells. It is well documented that transforming growth factors (TGF) in conjunction with hormones such as estrogen and PRL play a major role in modulating the proliferation and apoptosis of mammary cells. We first investigated the relationships between hPRL/hPRL-G129R and TGFs. We show that hPRL is able to down-regulate TGFß1 (apoptotic factor) secretion and up-regulate TGFα (survival factor) secretion in a dose-dependent manner in T-47D cells. More importantly the hPRL antagonist up-regulates TGFß1 and down-regulates TGFα secretion. When hPRL-G129R was applied together with hPRL, it blocked the effects of hPRL. Secondly, we tested the possible involvement of caspases in hPRL-G129R induced apoptosis. We have shown that caspase-3 is activated by hPRL-G129R at a concentration of 250 ng/ml in T-47D breast cancer cells. Thirdly, we explored the additive effects of an anti-neoplastic drug, cisplatin, with the hPRL-G129R in T47D breast cancer cells. We show that cisplatin and hPRL-G129R when applied together resulted in about 40% growth inhibition in T-47D cells.

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Abbreviations: hPRL, human prolactin; CDDP, cis-diammine-dichloroplatinum; FBS, fetal bovine serum; CSS, charcoal stripped serum; ATCC, American Type Culture Collection; TGFα and TGFß, transforming growth factor α and ß; ELISA, enzyme linked immunosorbent assay

Key words: prolactin antagonist, breast cancer, apoptosis, caspase-3, transforming growth factors, cisplatin

Introduction

Human prolactin (hPRL) has been shown to be one of the important survival/growth factors that can mediate the proliferation of breast cancer cells in an autocrine/paracrine manner. hPRL has been linked to breast cancer by several lines of evidence: a) biologically active PRL has been found in breast cancer cells (2); b) hPRL receptor expression levels are up-regulated in breast cancer cells/tissues (3); c) PRL transgenic mice have a high breast cancer rate (4); and d) a hPRL antagonist inhibits the proliferation of breast cancer cells by induction of apoptosis as demonstrated in our previous studies (1). These finding join the growing body of evidence that PRL is indeed one of the major players in the genesis/progression of breast cancer. In this study we investigate the possible mechanism(s) of hPRL-G129R induced apoptosis in breast cancer cells.

Apoptosis is a genetically regulated process of cell death and is an integral part of the development and homeostasis of all organisms. The mammary gland apoptosis occurs in sequential waves during development and involution beginning with each pregnancy and ending with each weaning. The regulation of normal breast development is dependent on hormones such as estrogen (E2) and PRL. In addition, growth factors such as TGFß and α are also implicated in the development of the breast. After weaning, withdrawal of PRL (along with other factors) results in one of the dramatic examples of apoptosis: remodeling of the breast that accompanies post-lactational involution. This highly regulated balance between proliferation, differentiation, and regression (apoptosis) requires fine control by hormones and growth factors, as well as cross-talk between epithelial cells and stromal fibroblasts of the mammary gland (5). In transgenic mouse studies, overexpression of TGFα blocks the mammary gland remodeling process, suggesting that TGFα may be acting as a survival factor for the mammary epithelium (6). In contrast, transgenic mice that overexpress TGFß showed increased mammary epithelium apoptosis throughout mammary development, suggesting that TGFß may be acting as an apoptotic factor for the mammary epithelium (6). Hormones such as PRL and E2 have also

been reported to modulate and cross-talk with the TGFs. For instance, TGFα has been shown to activate the mouse mammary tumor virus long terminal repeat in a similar fashion as PRL (7). E2 stimulates the secretion of TGFα and reduces the levels of TGFß1 in breast cancer cells (8,9) and PRL has been shown to inhibit the activity of TGFß in a murine hybridoma model (10). Interestingly, it has also been reported that TGFß1 inhibits PRL synthesis in the lactotroph cells through an autocrine/paracrine mechanism (11).

On the other hand, tamoxifen (TAM), an estrogen receptor (ER) antagonist, up-regulates TGFß1. This induction of TGFß1 is believed to play an important role in TAM induced apoptosis in breast cancer cells (12). In addition, plasma levels of TGFß are increased in women treated with TAM, an effect that appears correlated with its anti-tumor effects (5). TGFα is down-regulated by pure ER antagonists such as ICI 182,780 (13). Taken together, in mammary epithelial cells, TGFß acts as an apoptotic factor as it can be up-regulated by anti-cancer drugs and TGFα acts as a survival factor as it can be up-regulated by hormones that promote breast cancer cell proliferation such as E2 and PRL. Therefore any anti-breast cancer drug that can differentially modulate TGFs, specifically by up-regulation of TGFß (an apoptotic factor) and down-regulation of TGFα (a survival factor) could be very valuable in breast cancer therapy.

Cells undergoing apoptosis exhibit shrunken pyknotic nuclei as well as other characteristic changes such as blebbing. Molecular analyses of apoptotic cells can demonstrate characteristic DNA fragmentation, activation of specific 'death inducing' cellular genes and specific cellular proteases called caspases (14). These changes almost invariably involve chromatin condensation and its margination at the nuclear periphery, extensive double-stranded DNA fragmentation, and cellular shrinkage and blebbing. There is evidence that caspases contribute to the drastic morphological changes of apoptosis by proteolysing and disabling a number of key substrates, including the structural proteins gelsolin, PAK2, focal adhesion kinase, and rabaptin-5. Caspase-3 is one of the key caspases involved in DNA fragmentation (15). Caspase-3 initiates apoptotic DNA fragmentation by proteolytically inactivating DFF45 (DNA fragmentation factor-45)/ICAD (inhibitor of caspase-activated DNase), which releases active DFF40/CAD (caspase-activated DNase), the inhibitor's associated endonuclease. Thus, caspase-3 is the primary inactivator of DFF45/ICAD and therefore the primary activator of apoptotic DNA fragmentation (16). In view of the pivotal role played by caspase-3 in DNA fragmentation we wanted to determine if caspase-3 activation plays a part in hPRL-G129R induced apoptotic DNA fragmentation in breast cancer cells.

In our previous study (1) we demonstrated that the efficacy of growth inhibition of breast cancer cells was almost doubled when tamoxifen (an anti-estrogen agent) was combined with hPRL-G129R (an anti-prolactin). In this study we explored the *in vitro* effects of combining cisplatin, an anti-neoplasic chemotherapeutic drug along with hPRL-G129R as a potential combination therapeutic strategy. Cisplatin is a platinum-containing broad activity anti-neoplastic and alkylating agent effective against malignancies of the testes, ovaries, bladder, oesophagus, head and neck and lung (17). Recently, cisplatin has been reported to have a number of important therapeutic characteristics and has been used in combination therapy regimens. For example, cisplatin has been shown to immunosensitize tumor cells to Fas mediated apoptosis (18). Another study concluded that combination therapy with cisplatin and herceptin, a humanized monoclonal body directed against HER2, results in significant antitumor activity with the potential for reducing toxicity in metastatic breast cancer patients (19). Cisplatin has also been shown to improve the efficacy of gene therapy in malignancies of the head and neck, ovary, prostate and breast (20).

Materials and methods

Cell culture. The T-47D and MCF-7 cell lines obtained from ATCC are positive for both ER and PRL receptors. T-47D cells were grown in RPMI 1640 (phenol red-free to avoid its potential estrogen-like activities) supplemented with 10% fetal bovine serum (FBS, Gibco BRL) and ATCC recommended supplements. MCF-7 cells were grown in DMEM (phenol red-free) supplemented with 10% fetal bovine serum (FBS, Gibco BRL) and ATCC recommended supplements. Both cell lines were grown at 37°C in a humidity controled atmosphere in the presence of 5% CO_2.

Co-culture experiment. The cell proliferation assay was designed to take advantage of stable mouse L cell lines established by us that produce hPRL-G129R. Increasing numbers of L cells (or L-hPRL-G129R cells) in a range of 4,500-27,000 cells/well were co-cultured with a fixed number of MCF-7 cells (9,000/well) in 96-well plates. Simultaneously, a corresponding set of L cells (or L-hPRL-G129R cells) was cultured in a fixed volume of 200 µl in the same plate (without co-culture with MCF-7 cells) as background control. We have previously (1) used this co-culture set-up with T-47D cells. The total volume of the co-culture was 200 µl. The concentrations of hPRL-G129R at the end of 72 h co-culture were measured at 20-200 ng/ml - a concentration that is within the physiological range. Following 24-h, 48-h, or 72-h incubation, MTS-PMS solution (Cell Titer 96 Aqueous kit, Promega Corp. Madison, WI) was added to each well and plates were read at 490 nm using a Bio-Rad benchmark microplate reader. Seventy-two hours incubation time was optimal. The OD of MCF-7 cells was calculated as total OD (OD of MCF-7 plus L, or L-hPRL-G129R cells, respectively) minus the background ODs (L, or L-hPRL-G129R cells alone).

Caspase-3 assay. The breast cancer cells were switched from 10% FBS to 10% charcoal stripped serum (CSS) containing growth media 6 days before the assay. Approximately 2 million breast cancer cells were plated in 10% CSS containing medium growth media. The next day treatments were performed in 1% CSS containing growth media using hPRL-G129R prepared in the lab as described previously (1). A caspase-3 assay kit (ApoAlert CPP32/caspase-3 assay kit-Clontech Corp.) was used to assess the caspase-3 activity colorimetrically using the cell lysates. The specificity of the reaction was verified using a caspase-3 inhibitor (DEVD-fmk).

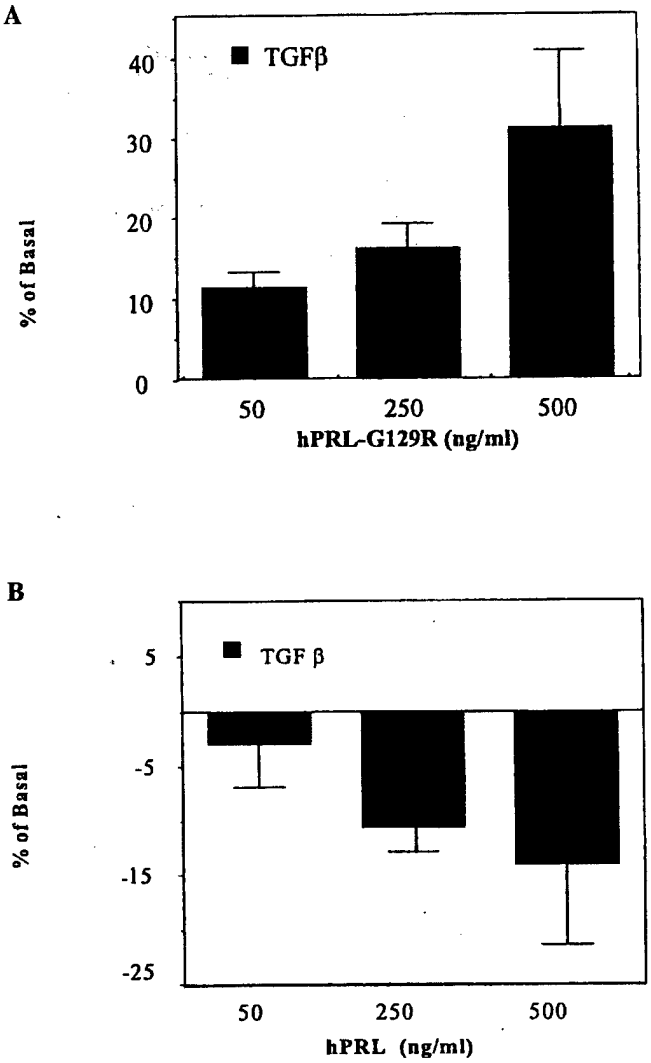


Figure 1. Modulation of TGFß1 by hPRL-G129R (A) and hPRL (B) in T-47D breast cancer cells. Cells were treated with 50 ng, 250 ng and 500 ng/ml of hPRL-G129R or hPRL for 72 h. The induction of TGFß1 by hPRL-G129R is expressed as percent of control above the basal level (A) and the inhibition of TGFß1 is expressed as percent of control below the basal level (B). Each data point represents the mean of at least three experiments. Bars, SD.

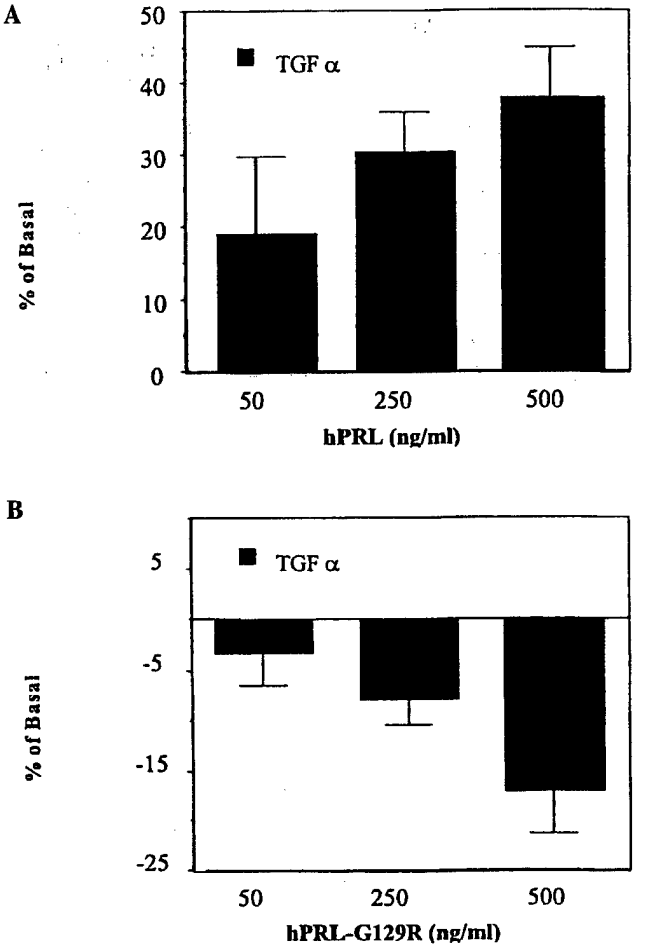


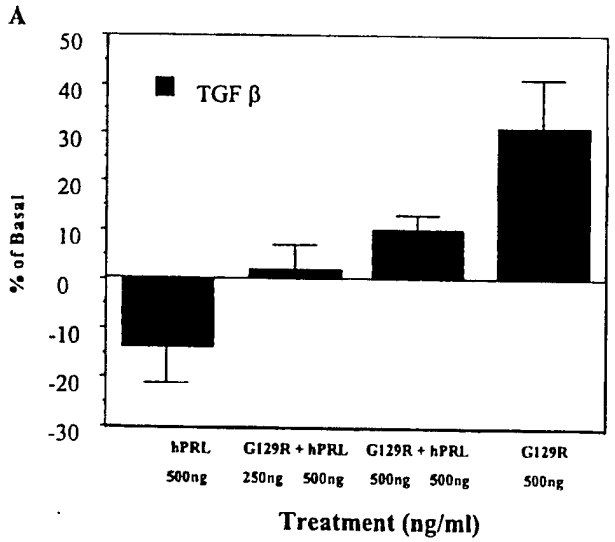
Figure 2. Modulation of TGFα by hPRL (A) and hPRL-G129R (B) in T-47D breast cancer cells. Cells were treated with 50 ng, 250 ng and 500 ng/ml of hPRL for 72 h. The induction of TGFα by hPRL is expressed as percent of control above the basal level (A) and the inhibition of TGFα by hPRL-G129R (B) is expressed as percent of control below the basal level. Each data point represents the mean of at least three experiments. Bars, SD.

ELISA-TGFβ1 and TGFα. Cells were plated in 12-well plates (Corning Costar) using 10% CSS containing growth medium. The following day the cells were starved using serum-free growth medium. Treatments were performed under serum free conditions using hPRL (kindly provided by Dr Parlow, National Hormone & Pituitary Program, NIH) and hPRL-G129R prepared in the lab as described previously (1). The supernatants were collected after a 72-h treatment and were stored as per the manufacturer's instructions. The ELISA's for both TGFß1 and TGFα were carried out using the supernatants from the same experiments. The TGFß1 kit was obtained from Promega Corp. (Madison, WI) and the TGFα kit was obtained from Oncogene Research Products (San Diego, CA).

Cell proliferation assay. The breast cancer cells were switched from 10% FBS to 10% CSS containing growth medium 6 days before the assay. For an individual cell proliferation experiment, 15,000 cells/well were plated in a 96-well plate cultured in 100 µl RPMI-1640 media containing 1% CSS (Collaborative Research, Bedford, MA). Cells were allowed to attach for 12 h, then an additional 100 µl of media containing varying concentrations of hPRL-G129R and cisplatin were added. The hPRL-G129R was prepared as described previously (1). After incubation, MTS-PMS solution was added to each well as per the manufacturer's instructions at 72 h. Plates were read at 490 nm using a Bio-Rad benchmark microplate reader (Bio-Rad Lab., Hercules, CA). Each experiment was carried out in triplicate and was repeated three to six times.

Results

Modulation of transforming growth factors α and β1 by PRL/hPRL-G129R in breast cancer cells. A dose-dependent increase in TGFß1 production was observed with the addition of hPRL-G129R in T-47D cells (Fig. 1A). At a maximal dose of 500 ng/ml, hPRL-G129R increased TGFß1 production to approximately 40% above the basal level (Fig. 1A). A dose-dependent decrease of TGFß1 was observed with the addition of hPRL (Fig. 1B). On the other hand, a dose-dependent increase in TGFα production was induced by



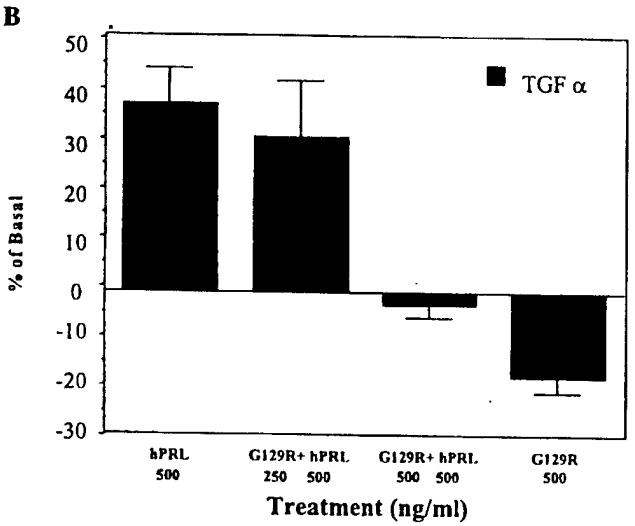


Figure 3. Competitive modulation of TGFß1 and TGFα by hPRL-G129R in T-47D breast cancer cells. Cells were treated for 72 h with a combination of hPRL-G129R and hPRL at a 1:2 and 1:1 ratio. The modulation of TGFß1 (A) or TGFα (B) is expressed as percent of control either above or below the basal level. Each data point represents the mean of at least three experiments. Bars, SD.

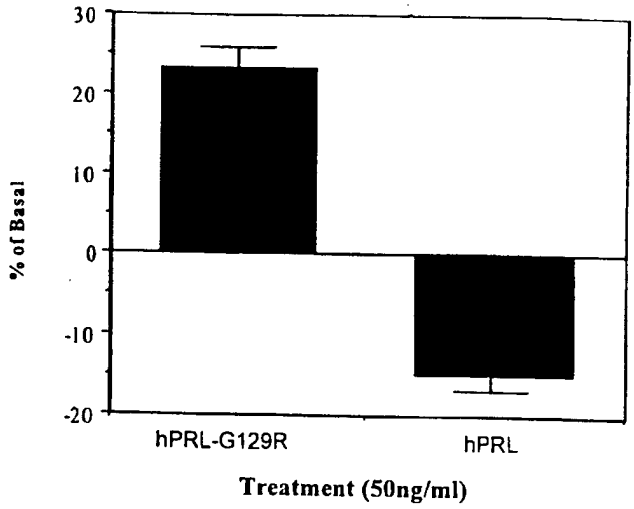


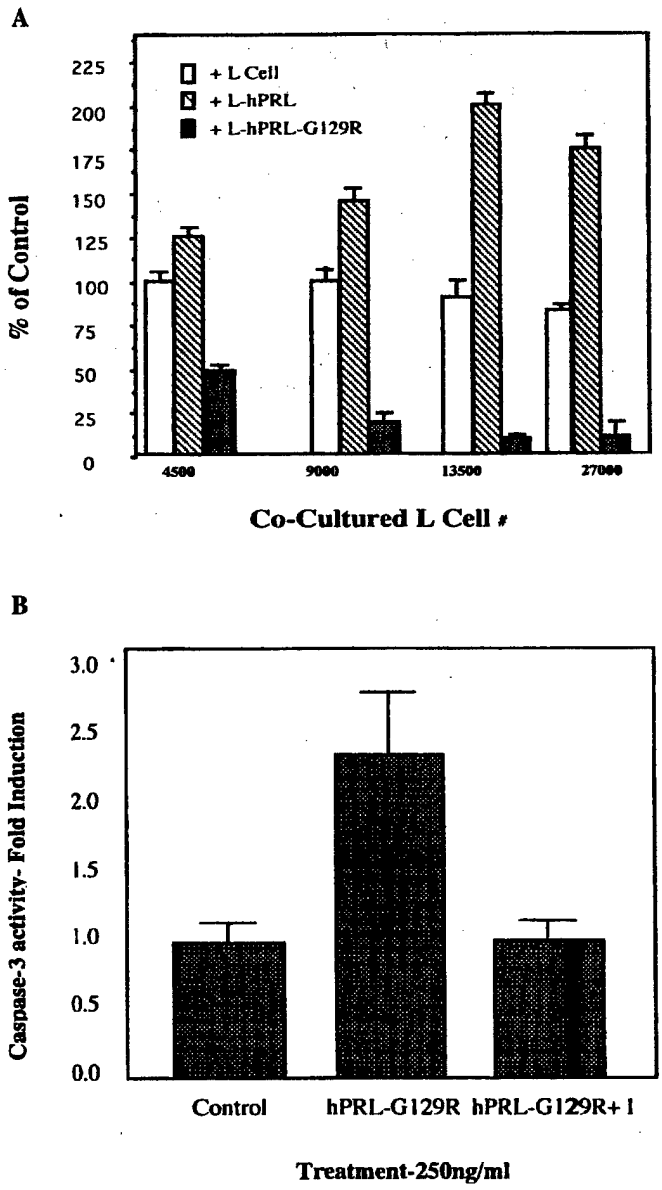
Figure 4. Modulation of TGFß1 by hPRL-G129R and hPRL in MCF-7 breast cancer cells. Cells were treated with 50 ng, of hPRL-G129R or hPRL for 72 h. The induction of TGFß1 by hPRL-G129R is expressed as percent of control above the basal level and the inhibition of TGFß1 is expressed as percent of control below the basal level. Each data point represents the mean of at least three experiments. Bars, SD.

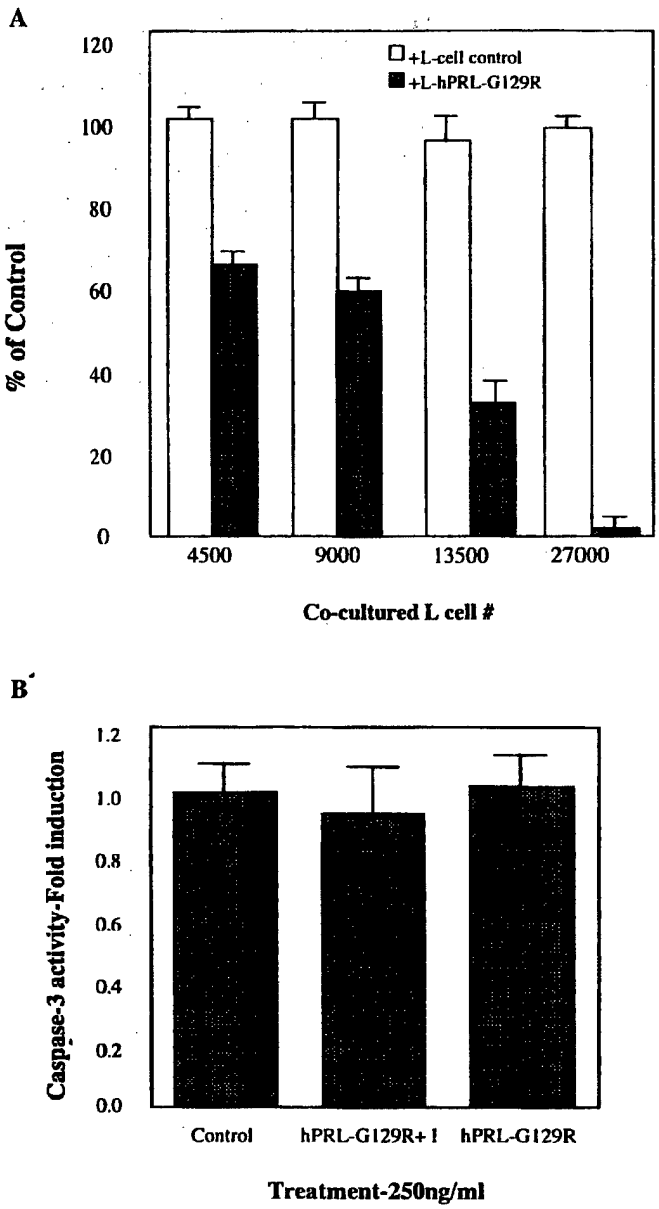
hPRL (Fig. 2A). The maximal increase of TGFα production was approximately 45% above the basal level (Fig. 2A). However, a dose-dependent decrease in TGFα production occurred when T-47D cells were treated with hPRL-G129R (Fig. 2B). The maximal inhibition of TGFα production was approximately 20% below the basal level (Fig. 2B). The TGFß1 level doubled when the concentration of hPRL-G129R was increased from 250 ng/ml to 500 ng/ml in the presence of a fixed amount of 500 ng/ml hPRL (Fig. 3A). In contrast, the TGFα levels were decreased by approximately 40% when the concentration of hPRL-G129R was increased from 250 ng/ml to 500 ng/ml in the presence of a fixed amount of 500 ng/ml hPRL (Fig. 3B). We observed the same trend in TGFß1 modulation in MCF-7 cells (Fig. 4) but surprisingly TGFα was not modulated by hPRL or hPRL-G129R (data not shown) in MCF-7 cells.

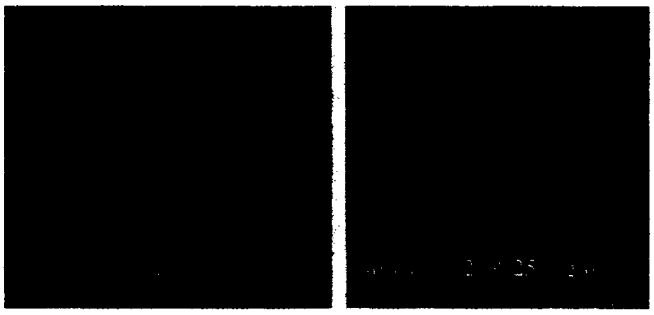
hPRL-G129R induced caspase-3 activation in T-47D breast cancer cells. The human prolactin antagonist, hPRL-G129R induces the activation of caspase-3 in T-47D cells at a dose of 250 ng/ml after 2 h treatment (Fig. 5B). Caspase-3 activity is approximately three-fold higher than that of untreated control. The specificity of caspase-3 activation was verified by adding a caspase-3 inhibitor DEVD-CHO along with hPRL-G129R (250 ng/ml). The inhibitor brought the level of caspase-3 activity to the level of control indicating that hPRL-G129R specifically inhibits caspase-3. For the purpose of comparison, we have also shown hPRL-G129R mediated inhibition of cell proliferation (Fig. 5A) and apoptosis (Fig. 5C) from our previous work (1).

Status of caspase-3 activation in MCF-7 breast cancer cells. Previously (1) we showed that hPRL-G129R were induced in both T-47D and MCF-7 cells (Fig. 6C). In view of the fact that caspase-3 was not activated by hPRL-G129R in MCF-7 cells (Fig. 6B), we wanted to determine if inhibition of cell proliferation by hPRL-G129R could also be observed in MCF-7 cells. MCF-7 cells were co-cultured with L cells expressing hPRL-G129R and as a control the MCF-7 cells were co-cultured with untransfected L cells. This co-culture system was used in T-47D cells in our previous work (1). The results (Fig. 6A) show that the L-hPRL-G129R cells are able to inhibit the proliferation of MCF-7 cells in a dose-dependent manner and at the highest dose, a near total inhibition was achieved.

Dose-response inhibitory effects of hPRl-G129R and its additive effects with cisplatin in breast cancer cells. Results from the cell proliferation assay in T-47D cells (Fig. 7) indicate that at a maximal dose of hPRL-G129R there is a 15% inhibition as compared to 25% inhibition with maximal dose of cisplatin. But when both cisplatin and hPRL-G129R







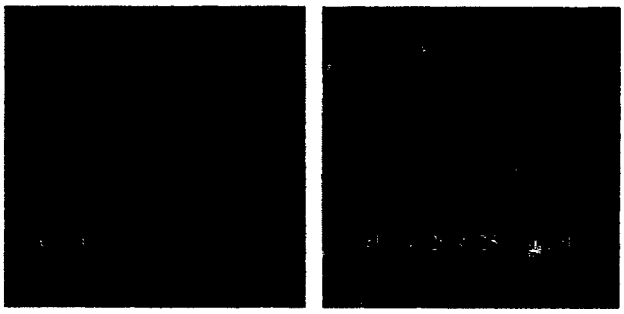


Figure 5. Caspase-3 activation in T-47D breast cancer cells. Induction of Caspase-3 activity by 2 h treatment with 250 ng/ml of hPRL-G129R in T-47D (B). The specificity of induction was verified by using a caspase-3 specific inhibitor DEVD-CHO (represented as I in the graph). For comparison hPRL-G129R mediated cell proliferation inhibition (A) and apoptosis (C) are shown. The data in A and C is from our previous work (1). Each data point represents a mean of at least three independent experiments with triplicates. Bars, SD.

Figure 6. Status of caspase-3 activation in MCF-7 breast cancer cells. Induction of caspase-3 activity by 2 h treatment with 250 ng/ml of hPRL-G129R in MCF-7 (B). The specificity of induction was verified by using a caspase-3 specific inhibitor DEVD-CHO (represented as I in the graph). Fig. 6A shows dose-response inhibitory effects of hPRL-G129R in MCF-7 human breast cancer cells using co-culture method. The x-axis represents the co-cultured L-hPRL-G129R cell numbers. For comparison hPRL-G129R mediated cell proliferation inhibition (A) and apoptosis (C) are shown. The data in C is from our previous work (1). Each data point represents a mean of at least three independent experiments with triplicate wells. Bars, SD.

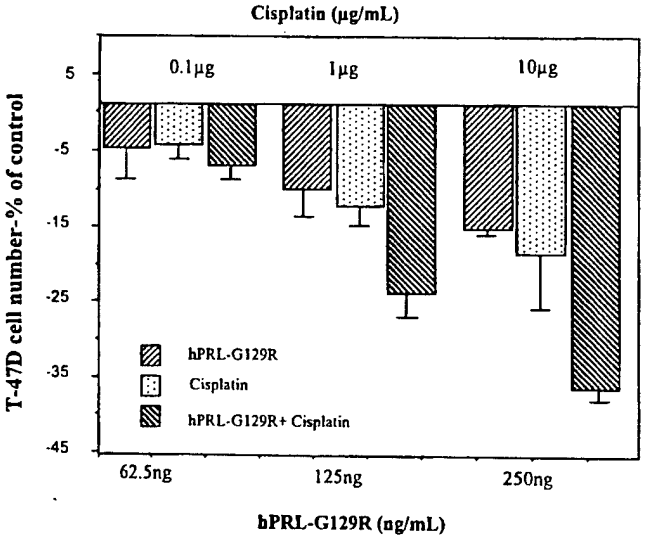


Figure 7. Inhibitory effects of hPRL-G129R and its additive effects with cisplatin in T-47D human breast cancer cell proliferation assay. The x-axis represents the hPRL-G129R concentration either in the absence or presence of cisplatin. Each data point represents a mean of at least three independent experiments with triplicate wells. Bars, SD.

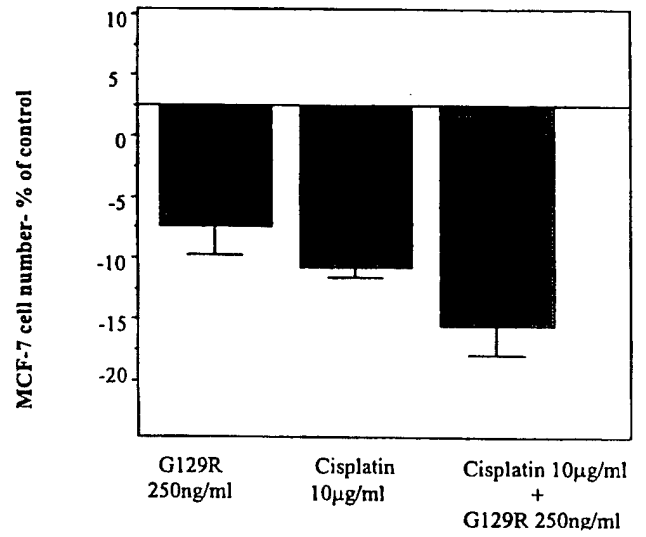


Figure 8. Inhibitory effects of hPRL-G129R and its additive effects with cisplatin in MCF-7 human breast cancer cell proliferation assay. The x-axis represents the hPRL-G129R concentration either in the absence or presence of cisplatin. Each data point represents a mean of at least three independent experiments with triplicate wells. Bars, SD.

were combined at their respective maximal doses the inhibition of T-47D cells reached about 40%. The same trend was observed in MCF-7 cells (Fig. 8). Thus, the inhibitory effect of cell proliferation by hPRL-G129R and cisplatin appears to be additive.

Discussion

In our previous study we demonstrated that hPRL-G129R, inhibited the proliferation of breast cancer cells through induction of apoptosis (1). In this study we explored the possible mechanisms of hPRL-G129R-induced apoptosis in human breast cancer cells, in particular, the roles of TGFs and caspase-3. In addition, we studied the potential additive effects of hPRL-G129R and cisplatin.

In view of the critical role played by TGFs in cell proliferation and apoptosis during mammary gland development (5) and their modulation by anti-estrogens (12,13) we studied the relationship between PRL, hPRL-G129R and TGFs. In this report, we demonstrate that hPRL-G129R up-regulates TGFß1 (an apoptotic factor) and down-regulates TGFα (a survival factor) after a 72 h treatment in T-47D cells (Figs. 1A and 2B) which is opposite to the effects elicited by the treatment of PRL (Figs. 1B and 2A). It is also noteworthy that the pattern of regulation of TGFs by the PRL antagonist, hPRL-G129R, in breast cancer cells is similar to that of anti-estrogens (12,13). Not surprisingly, E2 and PRL were reported to have a similar pattern in modulating TGFs (7-11). In order to assess the competitive nature of hPRL-G129R and hPRL in modulating the TGFs, T-47D cells were treated with increasing amounts of hPRL-G129R in the presence of a constant amount of hPRL. It is evident that hPRL-G129R is able to completely block and partially reverse the effects of hPRL (Fig. 3).

We speculate that the constant presence of PRL in the breast tumor microenvironment is responsible for TGFα up-regulation and TGFα down-regulation and the combination of these two events leads to increased proliferation and decreased apoptosis of breast cancer cells. The addition of hPRL-G129R competitively blocks the effects of PRL, thereby resulting in up-regulation of TGFß and down-regulation of TGFα leading to increased apoptosis and decreased proliferation of breast cancer cells. Although further studies are needed to elucidate the molecular mechanism(s) of hPRL-G129R modulation of TGFs in breast cancer cells, we postulate that there could be cross talk between the signal transduction pathways of PRL and TGFs at the levels of STATs (signal transducers and activators of transcription) and SMADs. STATs and SMADs are two families of transcription factors that are activated in response to respective ligand binding to their membrane bound receptors (21). In hematopoietic cells it has been shown that cytokine signaling through JAK/STAT pathways are generally antagonistic to TGFß, which signals through the SMAD pathway (22). A recent study has shown that IFNγ (a cytokine that signals through JAK/STAT pathway) inhibits the TGFß induced phosphorylation of SMADs in leukemia and fibrosarcoma cells (21). In this study we have shown that PRL, which signals through the JAK/STAT pathway, is able to inhibit TGFß production, suggesting that PRL might be antagonizing TGFß signaling through the inhibition of SMAD phosphorylation. On the other hand TGFα and PRL have both been shown to activate STAT3 in breast cancer cells (Cataldo LA *et al*, and Kelly PA *et al*, p173; Endocrine Society Meeting, 2000). In the present study we show that hPRL up-regulates TGFα production in breast cancer cells, suggesting that the PRL induced STAT3 activation might be involved in TGFα up-regulation.

In our previous study (1) we demonstrated that hPRL-G129R inhibited T-47D cell proliferation (Fig. 5A)

through the induction of apoptosis (Fig. 5C). In view of the pivotal role of caspase-3 activation in DNA fragmentation (16) we also investigated the role of caspase-3 activation in hPRL-G129R mediated apoptosis in breast cancer cells. We demonstrated that hPRL-G129R induces the activation of caspase-3 in T-47D cells (Fig. 5B) suggesting that caspase-3 might be involved in hPRL-G129R induced apoptosis in T-47D cells.

Breast cancer cells are a heterogeneous population of cell clones characterized by a number of biologic features (23). Our study corroborates this fact by demonstrating that MCF-7 cells differ from T-47D cells in their response to hPRL-G129R. For instance hPRL-G129R up-regulated TGFß and hPRL down-regulated TGFß in MCF-7 cells (Fig. 4) in a manner similar to that of T-47D cells. However we did not observe any modulation of TGFα by either hPRL or hPRL-G129R in MCF-7 cells (data not shown). Caspase-3 activation was not observed in MCF-7 cells (Fig. 6B) despite the fact that hPRL-G129R inhibits cell proliferation (Fig. 6A) and induced apoptotic DNA fragmentation in MCF-7 cells (Fig. 6C) as shown in our previous study (1). The lack of caspase-3 activation can be explained by a previous study that showed the caspase-3 gene to be defective in MCF-7 cells (15). The heterogeneity in breast tumors is one of the major reasons that tamoxifen is not universally effective even in estrogen receptor rich tumors (23). Combination therapy is becoming a viable strategy in combating tumor heterogeneity. A recent study (24) compared the efficacy of treatment with anti-estrogen (tamoxifen) alone as compared to a combination of a somatostatin analogue (octreotide), an anti-prolactin (CV205-502) and tamoxifen. The study concluded that the addition of a somatostatin analogue and an anti-prolactin might potentially enhance the efficacy of anti-estrogens in the treatment of breast cancer owing to favorable endocrine and possible direct anti-tumor effects. Previously we showed that we could almost double the efficacy of cell proliferation inhibition in T-47D cells by combining tamoxifen and hPRL-G129R (1). In the present study we investigated the effect of a non-endocrine related drug such as cisplatin and its additive/synergistic effects with hPRL-G129R. It is encouraging to note that the inhibitory effects of cell proliferation were more than doubled when hPRL-G129R and cisplatin were applied together in T-47D cells as compared to the maximal dose of hPRL-G129R (Fig. 7). This additive effect of hPRL-G129R and cisplatin was also observed in MCF-7 cells (Fig. 8).

In conclusion the mechanism of hPRL-G129R induced apoptosis might involve multiple factors including TGFα down-regulation, TGFß up-regulation and caspase-3 activation. The fact that hPRL-G129R shares similarities with TAM with respect to modulation of TGFs, together with our previous findings (1) strongly suggest that, it has the potential to be used by itself or in conjunction with TAM and/or cisplatin in breast cancer therapy. Our data also shows that the TGFα and TGFß could possibly be used as surrogate markers to monitor the therapeutic efficacy of hPRL-G129R. It is hoped that hPRL-G129R will emerge as a viable treatment option for breast cancer treatment.

Acknowledgments

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